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Effects of Anti-Anxiety Drugs and Other Anthropogenic Contaminants on Fathead Minnow Behavior and Brain Chemistry

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EFFECTS OF ANTI-ANXIETY DRUGS AND OTHER ANTHROPOGENIC CONTAMINANTS ON FATHEAD MINNOW BEHAVIOR AND BRAIN CHEMISTRY

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by
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Dr. Stephen Klaine, Committee Chair
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ABSTRACT

A majority of American adults in today's society are treated for anxiety with anti-anxiety medications. Some of these prescribed drugs are neither efficiently metabolized by the human body nor removed by wastewater treatment plants before they reach streams and rivers and so are often detected in the aquatic environment in trace concentrations. Once present in surface waters, they have the potential to cause adverse effects for aquatic organisms, including changes in both behavior and brain chemistry. Previous work has shown that upon exposure to 150µg/L fluoxetine, a selective serotonin reuptake inhibitor antidepressant, hybrid striped bass (*M. saxatilis* x *M. chrysops*) brain serotonin levels decreased by almost 50% over six days. This previous study also correlated reduced brain serotonin levels with behavioral alterations that decreased an organism's ecological fitness, specifically the ability of the organism to capture its prey. Similarly, other studies have suggested that fluoxetine, along with other anti-anxiety medications, including diazepam and buspirone, has the ability to alter an organism's anxiety behaviors. However, these studies failed to correlate the alteration in behavior with changes in brain neurotransmitter levels. While anxiety medications are designed to alter brain neurotransmitter levels, other investigators have suggested that other contaminants such as metals can also affect brain chemistry. A previous study has shown that carp exposed to sub-lethal levels of copper over the course of

one week experienced decreased brain serotonin levels in three different parts of the brain.

The current study characterized the effects of ethanol (positive control), fluoxetine, diazepam, buspirone, and copper exposure on brain chemistry and behavior in the fathead minnow (*Pimephales promelas*) using the standard light-dark anxiety behavioral bioassay. At the onset of the experiment, minnows exposed to ethanol, fluoxetine, and copper for one day displayed anxiolytic behaviors at lower concentrations. Longer exposures to buspirone and diazepam also elicited anxiolytic behaviors. Significant decreases in neurotransmitter levels were seen after exposure to ethanol, diazepam, and copper. In addition, my study further elucidated the relationship between brain chemistry and anxiety behaviors in fish, specifically the relationship between brain chemistry and minnow anxiety behavior after exposure to diazepam.

DEDICATION

I would like to dedicate this work to my parents, James and Margaret McLeod. They have blessed me with so much wisdom, love, and support during my academic career. They've always pushed me to be the best that I can be in everything that I do, and I know that I could never thank them enough for everything they have provided me throughout my life.

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Both Dr. Peter van den Hurk and Dr. Nishanth Tharayil allowed me to use their analytical instrumentation for my research. I used Dr. van den Hurk's HPLC for validation of my drug concentrations and his centrifuge for preparation of my

brain samples. He also allowed me to use his plate reader for my protein assays. Dr. Nishanth Tharayil developed a method for, and ran all my brain samples on, his LC-MS/MS for the detection of neurotransmitters. Without their generosity, I would not have been able to finish this research. In addition, Amith Maroli spent countless hours of his time helping me run the LC-MS/MS as well as with data analysis.

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be forever grateful to her for her tremendous help and support throughout my years here.

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CHAPTER ONE: INTRODUCTION

A large number of American adults are using prescribed medications to treat various anxiety disorders[1]. As the number continues to increase, concern about the concentrations of these medications in the aquatic environment also mounts as most major wastewater treatment plants do not effectively remove these compounds, which have been detected in many effluents[2,3]. Previous analytical methods have detected these drugs in the nanogram per liter to microgram per liter range in various surface water environments[4-6]. In addition to anti-anxiety medications and other pharmaceuticals, metals introduced by anthropogenic activities are also eliciting concern about the problems increased levels may cause in the environment. Copper is a metal both naturally found in the earth's crust and introduced anthropogenically via agricultural and mining practices[7]. Even present at trace concentrations, these compounds have been shown to alter organism behavior[8]. These changes in behavior may be linked to changes in the brain at sites where these compounds bind to serotonin (5-HT) and gamma-aminobutyric acid (GABA) receptors. The binding alters neurotransmitter concentrations in the brain, eliciting changes in organism behavior which can then lead to population level impacts in the aquatic environment.

My research focused on the effects of three anti-anxiety drugs (diazepam (**Fig. 1.1**), buspirone (**Fig. 1.2**), and fluoxetine (**Fig. 1.3**)) and copper on the brain

chemistry and behavior of the fathead minnow (*Pimephales promelas*). The light-dark behavioral bioassay, a standardized assay used to measure anxiety in both mammals and fish, was used to quantify minnow anxiety levels. Further, changes in brain chemistry resulting from exposure to these compounds were quantified by extracting the brains of exposed minnows and analyzing them for decreases in specific neurotransmitters. The testable hypotheses of my research included:

1. After exposure to either anti-anxiety medications or copper, fathead minnows will enter the light side of the tank more often and remain on the light side of the tank longer relative to controls.
2. Levels of 5-HT (**Fig. 1.4**), 5-hydroxyindoleacetic acid (5-HIAA) (**Fig. 1.**5) and GABA (**Fig. 1.6**) in the fathead minnow brain will decrease relative to controls after exposure to anti-anxiety medications and copper.

This research attempts to fill several gaps in the literature concerning the effects of anti-anxiety medications on the fish neurologic system. The goal of my research was to build on previous research conducted in our laboratory by examining two additional classes of compounds (*i.e.* anxiolytics and metals) and their effects on fish behavior and brain chemistry. This goal was achieved through completion of the following objectives:

1. Determine the effects of contaminants on fathead minnow anxiety behavior.
2. Determine the effects of these compounds on fathead minnow brain chemistry.
3. Correlate the changes in fathead minnow anxiety behavior after exposure to these compounds with changes in brain monoamine concentrations.

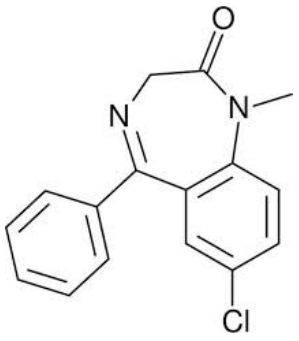

Figure 1.1 Chemical structure of diazepam (Valium®).

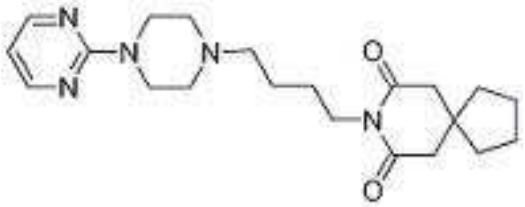

Figure 1.2 Chemical structure of buspirone (Buspar®).

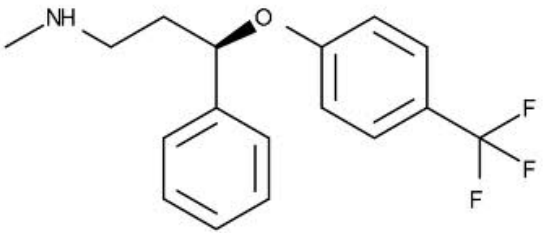

Figure 1.3 Chemical structure of fluoxetine (Prozac®).

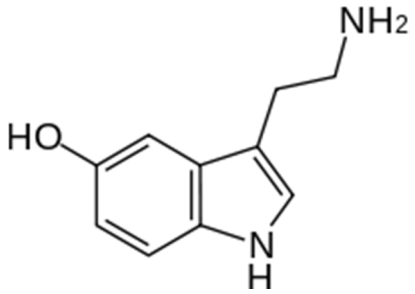

Figure 1.4 Chemical structure of serotonin (5-HT).

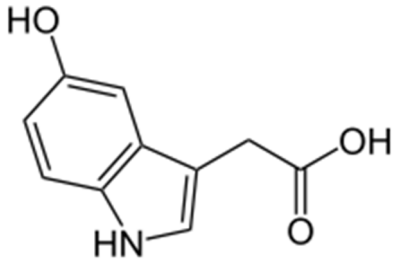

Figure 1.5 Chemical structure of 5-hydroxyindoleacetic acid (5-HIAA).

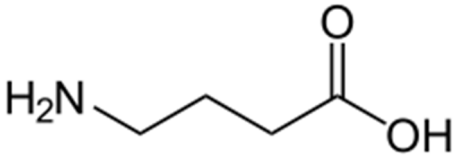

Figure 1.6 Chemical structure of gamma-aminobutyric acid (GABA).

CHAPTER 2: LITERATURE REVIEW

Environmental Occurrence of Pharmaceuticals and Anxiolytics

Recently, concern has been mounting about the occurrence of pharmaceuticals in the aquatic environment and their potential effects on the organisms that inhabit it. Little is known about the behavior and fate of these pharmaceuticals once they enter the aquatic environment[9]. As such, a significant amount of research has been done on pharmaceuticals in the aquatic environment. Concentrations of these drugs have been detected in surface waters in the nanogram per liter to microgram per liter range[4-6]. Nevertheless, even traces of these pharmaceuticals have been shown to alter organismal systems and animal behavior[8]. In addition, because these pharmaceuticals are being released constantly and in mixtures, the potential toxic effects are complex.

A proportion of the pharmaceuticals regularly detected in surface waters are medications prescribed to treat anxiety. These can include anxiolytics, drugs designed to alleviate anxiety, like diazepam (Valium®), buspirone (Buspar®), and chlordiazepoxide (Librium®), or antidepressants, like fluoxetine (Prozac®). Unlike the more widely used pharmaceuticals such as antidepressants and personal care products, little research has been performed and much less is known about the behavior and fate of anti-anxiety medications once they enter the aquatic environment.

Human use of these anxiety medications is a major point source through which the compounds contained in these medications enter the environment.

This source includes both consumption and direct disposal down the toilet[9]. Psychoactive pharmaceuticals, including anxiolytics, are one of the most commonly prescribed drug classes on a global scale, and the global annual production of these drugs is increasing to meet the demands of consumers[5]. According to the Anxiety and Depression Association of America (ADAA), about 13.3 million adults in the United States are treated for anxiety disorders, and many Americans take anxiety medications without a prescription[1]. These numbers are only expected to increase with time. Increases in both production and consumption of these drugs can lead to increased concentrations of both active and inactive metabolites in surface waters[10]. When taken into the body, a large majority of these compounds usually do not reach their intended target site and so are excreted in the active parent form[11]. Those compounds that do reach their target sites are neither completely nor efficiently metabolized by the body before being excreted as both active and inactive forms[10]. These compounds enter receiving streams, rivers, and lakes through the effluents of wastewater treatment plants that are not equipped to remove them[2,3]. They may be modified even further by microorganisms during the water treatment process or after, sometimes being transformed back into the parent form of the compound[12].

Once present in aquatic ecosystems, anxiolytics have the potential to cause a plethora of adverse effects on the environment and the organisms that live there. Although the concentrations of anxiolytics are usually not high enough (due to their relatively short half-lives) to cause a major toxic effect to fish and

other aquatic organisms, their continuous entry into the aquatic environment in mixtures is cause for concern. Behavioral changes that disrupt ecological fitness may occur due to the highly conserved mode of action of anxiolytics on the brain and neurotransmitter receptors. This situation has spurred much research concerning the toxicity of these substances to aquatic organisms.

Environmental Occurrence of Copper

Anti-anxiety medications and other pharmaceuticals are only one class of contaminants that are becoming increasingly prevalent in surface waters. Another class of contaminants that has the potential to disrupt the aquatic environment is metals, specifically copper. Copper is a naturally occurring metal in the Earth's crust and is found in surface waters as the bioavailable cupric ion (Cu^{2+}) where most of it is complexed or associated with organic matter[7,13]. Background concentrations of naturally occurring copper in freshwater systems range from 0.2 µg/L to 30 µg/L, but elevated levels as high as 74 µg/L have been widely reported[14,15]. Copper can also be found naturally in the air, associated with airborne particulates as well as in soils, where the mean concentration ranges from 5 to 70 mg/kg[13]. It is an essential metal required by all organisms in low concentrations (5-20 µg/g) with levels exceeding this range considered toxic[16].

In addition to being released naturally by volcanic eruptions, decaying vegetation, and forest fires, copper also enters the environment by anthropogenic

means including mining, agriculture, and manufacturing activities[7]. In 2001, an estimated 11,100,000 pounds of copper and 1,000,000,000 pounds of copper-containing compounds were released into the environment, with 0.4% and 0.04% of those totals being released directly into water, respectively[13]. It can only be assumed that these numbers have increased in more recent years. Most of the copper and copper-containing compounds released from all sources (including overburdens from copper mines and tailings, municipal sources, and pesticide use) in 2001 was to land (approximately 92% and 99.8% of those totals, respectively)[13]. Runoff and atmospheric deposition in areas where these processes are occurring as well as inefficient removal by wastewater treatment plants has caused significant water contamination in certain areas[17-19]. For example, estimated loading rates into surface waters from agricultural runoff ranged from 0.307 to 8.34 mg/hour in one area[13].

Once present in the aquatic environment, copper can easily bind to sediments and organic matter, thus increasing its persistence in the environment by becoming complexed to different environmental media. Bioconcentration can then occur in sediment dwelling organisms or be taken up by organisms living in the water column where toxic effects can occur, although there is little evidence that copper can biomagnify in the food chain[13,16]. Nevertheless, even trace concentrations of copper have the potential to affect higher organisms.

Mode of Action

Pharmaceuticals and Anxiolytics

Antidepressants and anxiolytics affect a number of neurotransmitters in the brain, including serotonin and GABA. These drugs, some of which are classified as selective serotonin reuptake inhibitors (SSRIs), can bind to and block reuptake transporters for these neurotransmitters (**Fig. 2.1**). Reuptake transporters are those on the pre-synaptic neuron that reabsorb specific neurotransmitters after the neural impulse has been transmitted. If blocked, the reuptake transporters can no longer reabsorb neurotransmitters, leading to an increase of those neurotransmitters (serotonin) in the synapses. Serotonin and GABA receptors in the vertebrate brain are highly conserved, and both serotonin and GABA play roles in many physiological and behavioral functions in fish[20]. Thus, when present in aquatic environments, these drugs have the potential to cause significant alterations in the behavior of aquatic organisms and in turn, affect their survival.

Anxiolytic compounds work by targeting the brain where they interact with neurotransmitter receptors, specifically the serotonin and GABA receptors. Serotonin is a monoamine neurotransmitter that is known to regulate mood, appetite, and sleep. Serotonin is found in neurons of organisms from all major clades, including vertebrates. Like other neurotransmitters, serotonin is released into the synaptic cleft during neuronal activity and binds to specific receptors, where it then elicits a response by activating ion channels to open and allow

charged ions through the plasma membrane. The flow of ions causes a change in the transmembrane potential of the neuron, which then sends an excitatory or inhibitory signal to the brain and other parts of the body. Serotonin may also be released outside the synapse. In this case, it exerts a more systemic effect on surrounding neurons[21]. GABA is thought to be the most important excitatory and inhibitory amino acid neurotransmitter in both the central nervous system (CNS) and peripheral nervous system (PNS)[22]and is believed to regulate fear and anxiety. It is widely distributed throughout the brain, found in 30-40% of all synapses[23]. In vertebrates, GABA binds to specific transmembrane receptors at inhibitory synapses in the brain. The process of ion exchange resulting from the binding of GABA is similar to that which results from the binding of serotonin. Once bound, ion channels open, causing an influx of negatively charged chloride ions into the cell or an efflux of positively charged potassium ions from the cell. The flow of ions results in a change in the transmembrane potential of the neuron, which ultimately sends an excitatory or inhibitory signal to the brain and other parts of the body.

As mentioned above, serotonin and GABA are not the only molecules that interact with their respective receptor systems. For example, there are several binding sites on the transmembrane GABA receptor including those for benzodiazepines, barbiturates, and steroids[24]. The mode of action of anxiolytic compounds is to interact with the receptor, either as agonists, chemicals that bind to a receptor and trigger a response, or as positive allosteric modulators,

chemicals that enhance the effect of an agonist. Diazepam is a positive allosteric modulator of GABA while buspirone is a known serotonin receptor agonist. Diazepam enhances the effect of GABA by binding to the benzodiazepine site on the $GABA_A$ receptor and increases the affinity of GABA for its receptor (**Fig. 2.2**). Buspirone is thought to bind preferentially to the 5-HT_{1A} receptor and reduce the synthesis and release of serotonin by acting as a partial agonist[25] (**Fig. 2.3**). Antagonists may also bind to the receptor, competing with either serotonin or GABA for binding and blocking their normal functions. Fluoxetine has been shown to bind to 5-HT_{2C} receptors and act as a competitive antagonist[74]. In fact, some of fluoxetine's therapeutic effects seem to be linked to this blockage of 5-HT receptors[74]. Studies performed on rats show that blockage of the GABA receptor and thus, of the signal, by antagonistic molecules increased anxiety-like behavior when the rats were observed in social interaction and conflict tests. Additionally, interaction of the GABA receptor with an agonist produced anxiolytic-like effects in the rats[26]. This mechanism works the same way in invertebrates, although GABA receptors in invertebrate organisms are thought to have less distinct pharmacological profiles and are less sensitive to some chemicals than are vertebrate receptors[27]. More is known about the specific mechanisms of the GABA receptor in the vertebrate system than in the invertebrate system. Additionally, more research has been performed on the mammalian system, which presents a research gap on the GABA receptor system in non-mammals, like fish. This is also the case with serotonin, although

anxiolytic drugs that act on the mammalian serotonergic system are believed to act in a similar manner on fear and anxiety behaviors in fish. Previous studies have confirmed this by measuring increased cortisol levels, an indication of activation of the stress axis[21].

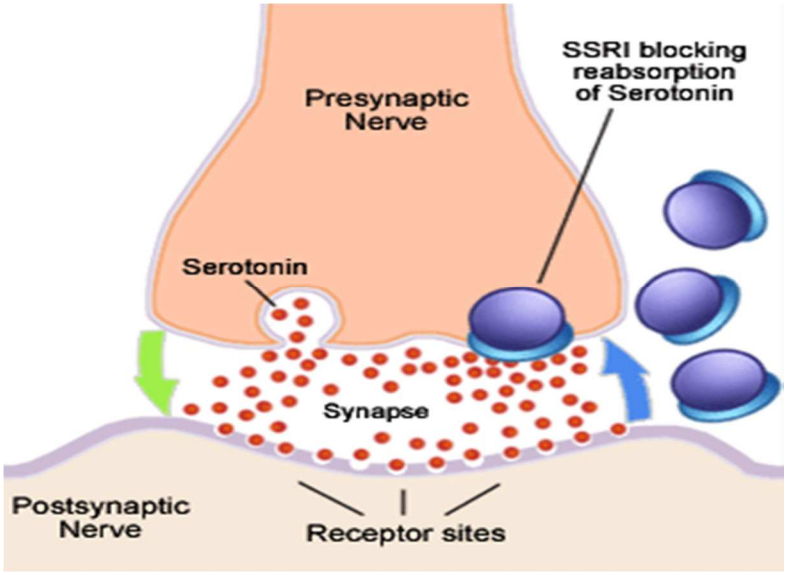

Figure 2.1 Mode of action of selective serotonin reuptake inhibitors (SSRIs). SSRIs, like fluoxetine, can bind to and block reuptake transporters on the pre-synaptic neuron, thus increasing the concentration of serotonin in the synapse.

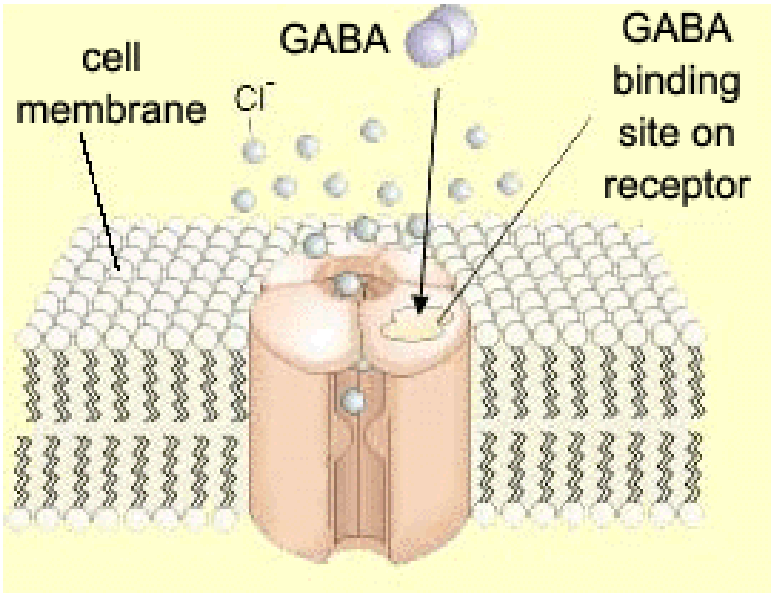

Figure 2.2 GABA receptor and the binding of GABA. Diazepam (Valium®) may bind to the benzodiazepine site on the $GABA_A$ receptor and increase the affinity of GABA for the receptor.

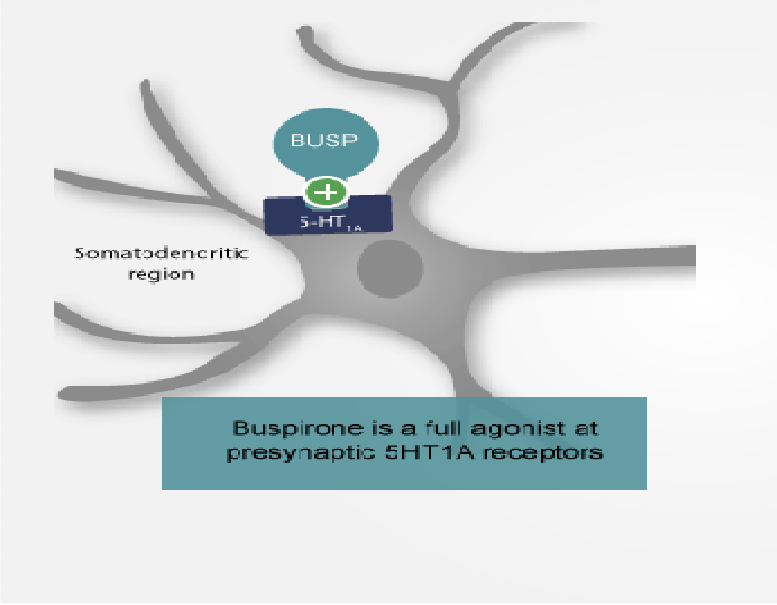

Figure 2.3 Mode of action of buspirone (Buspar®). Buspirone may bind to the 5-HT_{1A} receptor and reduce the synthesis and release of serotonin by acting as an agonist.

Copper

Copper is a well-known neurotoxicant and endocrine disruptor and has been shown to affect the serotonergic system in several ways. In fact, the serotonergic system in fish has been shown to be sensitive to various forms of copper-induced stress[28]. As such, exposure to copper can also have an effect on certain behavioral responses, although this subject has received much less attention[29,30]. Some of these behavioral responses can be explained by interference in normal function of the olfactory system in fish[29,31]. The olfactory system of fish is made up of epithelial tissues embedded with ciliated olfactory sensory neurons (OSNs). It is these OSNs that come into contact with the surrounding water and so are very sensitive to pollutants in the aquatic environment[32]. At low concentrations, copper diminishes the responsiveness of OSNs, leaving the fish susceptible to predation[33].

For aqueous copper exposure, the primary target organ is the gill epithelium, where copper inhibits the sodium potassium-ATPase (Na^+/K^+-ATPase) which leads to ionoregulatory imbalances and gill injury. Long-term exposure to copper can induce stress leading to activation of the hypothalamic-pituitary-adrenal (HPA) axis and cortisol release. This stress-induced stimulation

eventually results in catecholamine release, which leads to local increases in serotonin circulation. In contrast, studies have shown that dietary copper exposure may cause a failure in circulating serotonin. These opposing effects support the importance of route of exposure. In addition, there is evidence, although speculative, that copper affects the metabolism of serotonin, causing cross-linking and decreases in serotonin levels[34].

Although not as frequently studied as pharmaceuticals, metals have also been shown to affect monoamine neurotransmitter levels in the brain. One study exposed common carp to aqueous copper in the form of copper nitrate. The carp were exposed for one week. Scientists found dose-dependent decreases in serotonin in three different parts of the brain: the telencephalon, hypothalamus, and brain stem[28]. Lead causes the opposite effect, an increase in serotonin levels in the brains of catfish[35]. Similarly, when fathead minnows were exposed to aqueous lead for four weeks, researchers observed an increase in serotonin levels[35]. The lack of research that has been performed concerning the effect of metals on brain neurotransmitter systems further supports the need for my research as a bridge to span the gap.

The Importance of Behavior as an Endpoint in Ecological Toxicity Testing

Behavior is an important and informative endpoint in toxicity testing. Much of the concern over an increasing pharmaceutical presence in aquatic ecosystems is the effect of these compounds on organismal behavior, even at

trace concentrations[8]. Because concentrations of pharmaceuticals in the environment are usually lower than levels that cause significant acute and chronic toxicity, these behavioral endpoints have become quite useful as sensitive indicators of pharmaceutical effects on organisms[3,8,36,37].

To survive, an organism must be able to find and consume food, avoid predation, and successfully reproduce to pass on its genes to the next generation. If any of these behaviors is altered, an organism's chance of survival could significantly decline. Thus, chemicals like anxiolytics and antidepressants that alter fear and anxiety responses could potentially interfere with the behavior of an organism and cause its fitness to decline. Exposure may lead to anxiogenic-like, or increased anxiety-like behaviors. Organisms may be too anxious to interact socially with other organisms. For example, schooling behaviors may change or organisms may become reluctant to leave the safety of their shelter to find food and reproduce, negatively affecting their ability to survive. The opposite effect is seen in studies that explore the effects of neurotransmitter receptor agonists. These types of compounds cause an increased cellular signal that elicits anxiolytic effects, making organisms less anxious over exploring new environments and the approach of other organisms[26,38]. Organisms may ignore their basic survival instincts and disregard an approaching predator. Copper can affect basic behavioral functions like olfaction, or work by interfering with metabolic processes such as the breakdown of serotonin in the brain[30,34,39]. Because of this effect on serotonin, copper, like

anxiolytic drugs, has been hypothesized to affect anxiety behavior. All of the above situations thus have the potential to lead to effects on the population and even the ecosystem[40].

Various studies have investigated the effect of anxiolytics and copper on organism behavior in order to determine the sub-lethality of these contaminants. Feeding behavior is a common behavioral endpoint in toxicity testing. After exposure to sub-lethal concentrations of SSRI fluoxetine, hybrid striped bass exhibited a decrease in ability to capture prey. This concentration- and time-dependent decrease was correlated with depressed serotonin levels in the brain[37]. Similarly, hybrid striped bass exposed to venlafaxine exhibited an increased time to capture prey while simultaneously exhibiting decreased brain serotonin levels[36]. Similar research involved juvenile perch that were exposed to sertraline, an SSRI. The perch exhibited decreased foraging behaviors with increasing concentrations of sertraline[20].

Anxiety behavior is another behavioral endpoint that is commonly tested. Researchers exposed zebrafish to fluoxetine, buspirone, and diazepam and observed their behavior in the light-dark box scototaxis test. Chronic treatments with fluoxetine increased the time zebrafish spent in the white compartment while diazepam and buspirone produced similar effects[41]. The ability of fathead minnows to avoid predators was investigated after embryos were exposed to several common antidepressants at environmentally relevant concentrations,

either singularly or in mixtures. The C-start response, or escape reflex, of minnows was adversely affected by exposure to fluoxetine, venlafaxine, and bupropion singularly. Mixtures of these antidepressants also slowed predator avoidance behaviors in the minnows[42].

Reproductive behavior is yet another endpoint used in toxicity testing following exposure to pharmaceuticals. Concentrations of fluoxetine as low as 1 µg/L significantly affected male mating behaviors, specifically nest building and nest defending. At higher concentrations, males exhibited aggression, isolation, and repetitive behaviors[43]. Antidepressants have also been found to disrupt the reproductive system of molluscs, specifically spawning and larval release. Adverse reproductive effects following antidepressant exposure have also been discovered in amphipods and daphnids[44].

Coho salmon that were exposed to increasing, but sub-lethal concentrations of copper had an impaired sense of smell to natural odorants within 10 minutes of exposure. Researchers concluded that copper is broadly toxic to the salmon olfactory system[29]. A second study on copper focused on shoaling behavior in killifish. Shoaling occurs when fish congregate in groups for social reasons. Exposure to acute copper resulted in decreases in time to first shoaling and total time spent shoaling[30]. The above evidence confirms that important behavioral and physiological processes can be affected by chemicals in the environment and cause a decline in an organism's ability to survive,

stressing the importance of behavioral endpoints in ecological toxicity testing following exposure to environmental contaminants.

Quantifying Anxiety Behavior in Fish

Anxiety behavior is a very useful and informative endpoint that is becoming increasingly popular in toxicity testing. Because it is considered a sublethal endpoint, the effects of certain toxicants on this behavior can easily be observed and quantified. As such, several different bioassays have been developed to observe and quantify anxiety behavior in both mammals and fish.

Zebrafish (*Danio rerio*) are becoming increasingly promising as a model organism for stress and anxiety research. The potential for the use of this species has emerged quite recently. Zebrafish are useful because they possess all of the standard vertebrate neurotransmitters and serve as non-mammalian organisms that are physiologically homologous to humans. In addition, much is known about their genome and their neuroendocrine system responds well to stress in the form of physiological responses making them a useful species for behavioral bioassays[45,46]. The two most widely used behavioral assays that employ zebrafish as the model organism are the novel tank diving test and the light-dark scototaxis test. The novel tank diving test involves a tank divided into sections (top, bottom, etc.) and explores the zebrafish's instinctual response to seek shelter in unfamiliar environments by diving to the bottom of the tank and remaining there until the fish feels safe enough to explore. Researchers assess

anxiety by quantifying latency to enter the upper half and time spent in each compartment as well as specific behaviors, including erratic movements and freezing. The test relies on an exposure to novelty for quantifying anxiety responses in zebrafish, with longer latencies to enter the upper compartment, reduced time spent in the top compartment, increased erratic movements, and freezing as measures of increased anxiety. The light-dark scototaxis test, also known as the light-dark preference test, is similar in that it also relies on exposure to novelty. However, the tank is divided into equal light and dark or white and black compartments. It has been suggested in a previous study that zebrafish prefer a darker environment[47] and it is this preference on which the test is based. Entries to the light compartment and time spent in the light compartment per entry are quantified with increased entries and more time spent in the light compartment being indicative of decreased anxiety behavior. A third, less common behavioral assay is the open field test which consists of a large open space divided into inner and outer zones. The assay relies on the zebrafish to display behaviors associated with thigmotaxis, or avoiding the center of a novel area and moving in close proximity to the boundaries of said novel area[48]. Variations of these tests have also proven useful for investigating anxiety behavior in zebrafish as well as other organisms.

Zebrafish are usually exposed to certain anxiolytic or anxiogenic drugs before being subjected to one or both of these tests, although researchers have investigated the effects of restraint stress on zebrafish behavior instead of

chemical exposure[48]. These drugs can serve as positive controls for these behavioral bioassays, allowing researchers to investigate the effects of other compounds not usually used with these assays. Researchers administered a chronic treatment of fluoxetine (100 µg/L) to zebrafish over a period of two weeks and observed them in the novel tank diving test. The exposed fish displayed anxiolytic behaviors in the form of lower latency to enter the top compartment, more time spent in the top compartment, and a decreased number of erratic movements as compared to controls[45]. A second study investigated the effects of fluoxetine, buspirone, diazepam, and ethanol on zebrafish anxiety behavior using the light-dark scototaxis test. Fish were injected with 5 or 10 mg/kg fluoxetine, 25 or 50 mg/kg buspirone, or 0.02 or 0.2 mg/kg diazepam. Fish injected with 10 mg/kg fluoxetine spent more time in the light side of the tank while those injected with buspirone spent more time in the light side at both doses. Diazepam-injected fish also showed an anxiolytic effect. Additionally, fish that were exposed to 0.25% ethanol spent more time on the light side of the tank[41]. Other studies show similar results of anxiolytic compounds decreasing anxiety behavior in fish[49-51]. The effects of nicotine, cocaine, and caffeine have also been investigated using these behavioral tests[41,52,53]. Because of the validity and plasticity of these behavioral assays, the results may be applied to other areas including genetics and medicine, setting the scene for further advances in these fields[54,55].

Quantifying Neurotransmitters in Biological Samples

Several previous studies have been able to quantify the concentrations of neurotransmitters in biological samples from mammals, including humans, and from fish. Methods using various forms of chromatography: gas chromatography (GC), high performance liquid chromatography (HPLC), and liquid chromatography/tandem mass spectrometry (LC-MS/MS), have reported accurate and reliable results for the analysis of neurotransmitters, including serotonin and GABA. One study quantified levels of three monoamine neurotransmitters (dopamine, norepinephrine, and serotonin) as well as melatonin and its metabolite, 7D-melatonin, in the brains of sea lampreys (*Petromyzon marinus*) using an LC-MS/MS method with a reversed-phase column. Pre-treatment of samples included solid-phase extraction (SPE) to purify and extract the target compounds from samples. Researchers were able to quantify all five compounds with extreme accuracy and limits of detection of 0.14, 0.09, 0.07 ng/mL for norepinephrine, dopamine, and serotonin, respectively. The same study also investigated the stability of these compounds at 4°C in the dark. Serotonin and dopamine were stable for up to three days and one day, respectively, although norepinephrine was not found to be stable under these conditions. In addition, the accuracy of three different types of SPE cartridges was tested. Recoveries were highest for samples that were prepared using Bond-Elut C18 cartridges[56]. Another study utilized HPLC with fluorescence detection to analyze the concentrations of 5-HT and 5-HIAA in sea lamprey

plasma samples. SPE was performed with Oasis HLB cartridges. Limits of detection ranged from 0.04 to 0.13 ng/mL[57]. Using an HPLC method with electrochemical detection, GABA and dopamine levels were quantified in the hypothalamus of largemouth bass (*M. salmoides*) in the nmol/g range[58]. In addition to the latter, there have been other studies performed using both whole and partial fish brain samples in several different species, including the fathead minnow, goldfish, trout, and carp[28,59-61]. Finally, one study quantified free GABA in the cerebrospinal fluid of humans in the nmol/L range using an isocratic HPLC method with electrochemical detection[62].

With the recent advances in analytical analysis of biological samples, neurotransmitter detection methods are becoming more efficient and accurate. Researchers developed a method using LC-MS/MS to quantify GABA and glutamate in rat brain microdialysis samples with a limit of quantitation of 1 and 10 nM, respectively. The use of a hydrophilic interaction liquid chromatography (HILIC) column allowed researchers to separate the polar analytes from the sample matrix, and thus, the pre-treatment of samples was not required, making this method more efficient than previously reported methods. In addition, when compared with previous methods, this method has a higher sensitivity, is more selective, and is more rapid with a run time of three minutes[23]. A second study involving rat microdialysis samples was able to detect GABA and glutamate at even lower concentrations (0.03 and 0.8 pmol, respectively). However, in contrast to the previous study, researchers utilized an HPLC method which

allowed for detection of very low amounts of these neurotransmitters[22]. This evidence, along with many other studies, demonstrates the ability of researchers to efficiently and accurately quantify neurotransmitter levels in many types of biological samples.

Although behavioral research after chemical exposure is plentiful, only a few studies have actually correlated changes in behavior following chemical treatment to changes in brain chemistry in fish[21]. Previous research in our lab found a negative correlation between time to capture prey and brain serotonin activity in hybrid striped bass (*M. saxatilis* x *M. chrysops*) after exposure to fluoxetine[37]. With the completion of this research, I hope to also correlate changes in fish behavior, specifically anxiety behavior, and decreases in neurotransmitter activity.

CHAPTER 3: MATERIALS AND METHODS

Test Chemicals

Methanol, acetonitrile, glacial acetic acid, and triethylamine were all HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA). The nitric acid and hydrochloric acid used to acidify collected water samples were Trace Metal Grade and also purchased from Fisher Scientific (Fair Lawn, NJ, USA). The fluoxetine hydrochloride used in all experiments was generously donated by Fermion in Finland. Buspirone hydrochloride and copper (II) sulfate pentahydrate were purchased from Alfa Aesar (Ward Hill, MA, USA). Absolute ethanol was purchased from Aldrich (Ward Hill, MA, USA). The diazepam was purchased from Qualitest Pharmaceuticals (Huntsville, AL, USA) through Godley-Snell Research Center (Clemson, SC, USA). Water used for all analytical procedures was passed through a Milli-Q-Super-Q Filtration system (Millipore®, Billerica, MA, USA) so that it was ultra-purified and had a measured resistance of 18 mΩ·cm. Exposures were conducted in moderately hard reconstituted water that was prepared according to the US EPA recipe: 190 L Millipore® Milli-Q water, 11.4 g $CaSO_4$, 11.4 g $MgSO_4$, 0.76 g KCl, and 18.24 g $NaHCO_3$[63]. All salts for moderately hard water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Tricaine-S (MS-222) used for euthanizing minnows was purchased from Western Chemical (Ferndale, WA, USA.

Fish

Fathead minnows (*Pimephales promelas*) were either purchased from I.F. Anderson Minnow Farm (Lonoke, AR, USA) or caught in traps that were deployed in ponds at the Cherry Farm Aquatic Research Lab at Clemson University (Clemson, SC, USA). The minnows were held in 100 L holding troughs that were maintained as flow through systems, supplied with fresh water from Lake Hartwell (Clemson, SC, USA) (pH=6.28 ± 0.17, Hardness=24 mg/L as $CaCO_3$, Alkalinity=10 mg/L as $CaCO_3$). The water was filtered and sterilized before reaching the holding troughs. Water temperature was maintained within a range of 20-27°C. The water contained within the holding troughs was constantly aerated through airstones. During holding, minnows were fed a commercial diet of Tetramin® Tropical Flakes that were purchased from Dr.'s Foster & Smith, Inc. (Rhinelander, WI, USA). Minnows were transported to the Clemson University Institute of Environmental Toxicology (Pendleton, SC, USA) for all exposures and allowed to acclimate for at least 2 days in a 33 L (20" x 10" x 12 ½") holding aquarium. The holding aquarium was maintained as a recirculating system with a Tetra® Whisper® filter containing Ultra-activated® carbon (Blacksburg, VA, USA) and contained moderately hard reconstituted water according to the US EPA's recipe above. The holding aquarium was constantly aerated using an airstone, and minnows were fed the same commercial diet of Tetramin® Tropical Flakes purchased from Dr.'s Foster & Smith, Inc. (Rhinelander, WI, USA). Minnows chosen for exposures were approximately 5-6 cm in size.

Experimental Design

All exposures were conducted in an animal testing room at the Clemson University Institute of Environmental Toxicology (Pendleton, SC, USA). Exposure aquaria (2 L, 24 cm x 6.4 cm x 16.5 cm) were half covered with black plastic sheeting (Sunbelt Plastics, Monroe, LA, USA) to simulate a “light” environment and a “dark” environment (**Fig. 3.1**). A clear plastic cover was also half covered with the same black plastic sheeting and placed on top of each aquarium to prevent minnows from jumping out and also significant evaporation of the water. Each exposure aquarium was aerated with two airstones, one on the “light” side and one on the “dark” side to prevent minnows from preferentially choosing the side of the tank with the airstone. Twelve exposure aquaria (n=3 per treatment) were placed on a metal rack with three shelves so that there were four aquaria per shelf (**Fig. 3.2**). A white bed sheet was hung behind the metal rack to make filming and visual observation of each tank easier. Volumes of 1.8 and 0.9 L were measured and marked on each exposure aquarium to indicate the fill lines for exposure volume and half renewal volume on half-renewal days, respectively. Concentrations included a low, medium, and high treatment along with a control for each chemical and were randomly assigned to each exposure aquarium so that there were three replicate exposure aquaria for each of the four concentrations. After the acclimation period, minnows were transferred from the 33 L holding aquarium to the exposure aquaria so that there was one minnow per aquarium. Each exposure aquarium was spiked with the appropriate amount of

drug and subsequently re-spiked with half of the original amount on half renewal days according to the half-life of each drug. Stock solutions of each drug were remade for each exposure. Water samples were taken two hours after the initial spike on Day 0 and after each re-spike on renewal days to allow for equilibration. Water samples were acidified with the appropriate acid and stored at 4°C until further analysis. Acute exposures lasted for 24 hours while long-term exposures lasted for nine days. Time-points were taken at 30 minutes, 3 hours, 12 hours, and 24 hours after exposure for acute exposures and 30 minutes (Day 0), Day 3, Day 6, and Day 9 for long-term exposures so that there were a total of four time-points for each exposure. Time-points during long-term exposures were taken at the same time each day. For each time-point, minnow behavior was recorded with a video camera from outside the animal testing room so as not to disturb the minnows. The number of entries of each minnow to the light side and the duration of each entry was recorded. An "entry" was defined as any time when at least half of the minnow body was on the light side of the aquarium. During exposure, minnows were fed the same commercial diet of Tetramin® Tropical Flakes every day purchased from Dr.'s Foster & Smith, Inc. (Rhinelander, WI, USA). In addition, water quality measurements (temperature in °C, dissolved oxygen in mg/L, alkalinity as $CaCO_3$, hardness as $CaCO_3$, and pH) were recorded every day with a YSI™ 556MPS Meter and a ProODO™ Meter (YSI Incorporated®, Yellow Springs, OH, USA). During exposures, the testing room was not entered unless absolutely necessary, to avoid influencing the behavior of

the minnows. After the completion of each exposure, minnows were euthanized in a solution of MS-222 (Western Chemical, Ferndale, WA, USA), and their brains were extracted and stored in a -80°C freezer until further analysis. Due to limited sample size (n=3 per treatment), brains were only extracted after 24 hours for acute exposures and after nine days for long-term exposures.

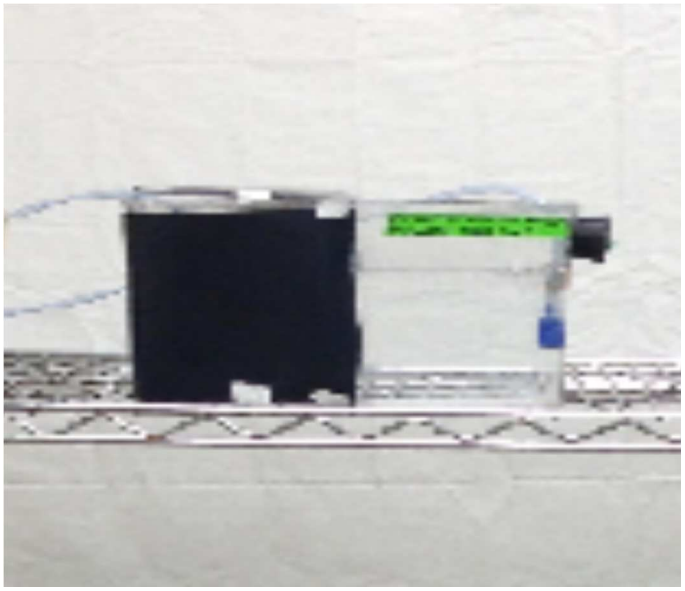

Figure 3.1 A single exposure aquarium with the left half covered with black plastic sheeting.

Figure 3.2 Exposure aquaria setup.

Diazepam, Buspirone, and Fluoxetine Half-Life Studies

The stabilities of diazepam, buspirone, and fluoxetine were investigated to determine if subsequent additions of the chemical would be necessary to maintain constant concentrations throughout the duration of the experiments. Prior to the initiation of exposures, a two-day stability experiment was performed. The same experimental setup as described above was used. A single minnow was added to each exposure aquaria to simulate the same conditions as during the actual experiment. Water samples were collected from each aquarium daily and measured to determine drug concentrations.

Ethanol Exposures

Absolute ethanol (Aldrich, Ward Hill, MA, USA) was used as a positive control to ensure the validity of the light-dark behavioral bioassay. In scientific experiments, the positive control is a treatment with a known response. Nominal concentrations chosen included 0.25%, 0.5%, and 1.0% (v.v.) along with a negative control. Half water renewals and re-spikes occurred every three days for long-term exposures. Renewals and re-spikes were not performed for acute exposures.

Diazepam, Buspirone, and Fluoxetine Exposures

Spiking solutions of diazepam were prepared by dissolving a 10 mg pill in 10 mL of methanol and placing on a stir plate to equilibrate for 24 hours. Spiking solutions of buspirone and fluoxetine were prepared by dissolving buspirone hydrochloride and fluoxetine hydrochloride in methanol. Concentrations of spiking solutions were selected so that there was no more than 0.1mg/L methanol in the exposure tanks. This value is the ASTM recommendation for methanol as the carrier solvent[73]. The control tanks were spiked with the highest volume of methanol that was calculated from desired exposure concentrations to ensure there was no toxicity due to the carrier solvent. Nominal concentrations chosen included 35, 75, and 150 µg/L along with the control. These concentrations were chosen because previous research in our lab discovered decreases in bass serotonin levels after exposure to similar concentrations of fluoxetine[36,37]. New spiking solutions were prepared for each exposure. The half-lives of diazepam, buspirone, and fluoxetine were determined to be about

one day. To maintain constant concentrations of these drugs throughout the duration of the experiment, re-spikes occurred every day for long-term exposures with appropriate volumes of diazepam, buspirone, and fluoxetine spiking solutions to reach nominal concentrations. To prevent the buildup of nitrogenous waste products, 50% of the aquaria water was renewed every day prior to re-spiking. Aquaria were then re-spiked with the appropriate volume of spiking solution, taking into account the half-life of each drug and the volume of water removed from the tank. Renewals and re-spikes were not performed for acute exposures. Water samples were taken two hours after the initial spike on Day 0 and after each respike on Days 3, 6, and 9 for verification of drug concentrations. Water samples were acidified with three drops of 2 M hydrochloric acid and stored at 4°C until further analysis.

Copper Exposures

Spiking solutions of copper were prepared by dissolving copper (II) sulfate pentahydrate in Milli-Q water. Nominal concentrations chosen included 20, 40, and 80 µg/L. These concentrations were modeled after previous research that observed decreases in brain serotonin levels in three different parts of carp brains after exposure to copper[28]. Because the carrier solvent for copper was Milli-Q water, nothing was added to control tanks. New spiking solutions were prepared for each exposure. Previous research determined that copper was relatively stable in this system and had a half-life of about three days (data not shown). To maintain constant concentrations of copper throughout the duration

of the experiment, re-spikes occurred every three days for long-term exposures with appropriate volumes of copper spiking solutions to reach nominal concentrations. To prevent the buildup of nitrogenous waste products, 50% of the aquaria water was renewed every three days prior to re-spiking. Aquaria were then re-spiked with the appropriate volume of copper spiking solutions, taking into account the stability of copper and the volume of water removed from the tank. Renewals and re-spikes were not performed for acute exposures. Water samples were taken two hours after the initial spike on Day 0 and after each respike on Days 3, 6, and 9 for verification of copper concentrations. Water samples were acidified with nitric acid and stored at 4°C until further analysis.

Verification of Diazepam, Buspirone, and Fluoxetine Concentrations

To confirm exposure concentrations, water samples were taken every day prior to re-spiking. Samples were collected in the appropriate volumetric flask and acidified with three drops of 2 M hydrochloric acid. Samples were extracted immediately on 3 mL HyperSep C-18 SPE cartridges with a bed weight of 500 mg (Thermo Fisher Scientific, Pittsburgh, PA, USA). Prior to extraction, cartridges were conditioned with 3 mL of acetone, 3 mL of methanol, and 6 mL of Milli-Q water. Samples were then loaded onto the cartridges. After the entire sample had passed through the cartridge, the cartridges were dried under vacuum for at least 30 minutes. Cartridges were eluted immediately with a solution of methanol and 1% acetic acid. Elute was collected and stored in sample vials at 4°C until HPLC analysis.

The HPLC system consisted of a Waters 1525 Breeze HPLC Pump, a Waters 717 Plus auto sampler, a Waters 2475 multi-wavelength fluorescence detector, and Waters 2487 multi-wavelength absorbance detector (Waters, Milford, MA, USA). A Varian Polaris 5 C-18A reverse phase analytical column (250 mm long, 4.6 mm I.D.) (Varian Inc., Lake Forest, CA, USA) was used to achieve chromatographic separation. The mobile phase for diazepam consisted of 60:40 HPLC grade acetonitrile: Milli-Q water adjusted to a pH of ~2 with glacial acetic acid and 0.1% triethylamine. The mobile phase for buspirone consisted of 35:65 HPLC grade acetonitrile: Milli-Q water adjusted to a pH of ~3 with glacial acetic acid and 0.1% triethylamine. The mobile phase for fluoxetine consisted of 40:60 HPLC grade acetonitrile: Milli-Q water adjusted to a pH of ~3 with glacial acetic acid and 0.4% triethylamine. All mobile phase solutions were filtered through a 0.45 µm nylon filter and degassed for at least 30 minutes using a sonication bath. The flow rate was 1mL/min and a 40 µL injection volume was used. For diazepam, the absorbance detector was set to 238 nm, and the retention time was approximately six minutes. The absorbance detector was set to 240 nm for buspirone while the retention time was approximately six minutes. For fluoxetine, the fluorescence detector was set at an excitation wavelength of 270 nm and an emission wavelength of 300 nm. The retention time for fluoxetine was approximately 11 minutes.

Verification of Copper Concentrations

To confirm exposure concentrations, water samples were taken every three days prior to re-spiking. Samples were collected in 15mL Falcon tubes (VWR International, West Chester, PA, USA), acidified with nitric acid, and stored at 4°C until ICP-MS analysis.

Preparation of Brain Tissue

When ready for analysis, brains were collected from storage in -80°C. Individual brains were weighed and transferred to new 1.5 mL centrifuge tubes (Eppendorf, Westbury, NY, USA). Brains were homogenized using a 400 W, 20 kHz Digital-Model 450 Sonifier (Branson, Danbury, CT, USA) in 30 µL of Milli-Q at 10% amplitude for five seconds. Three volumes (90 µL) of cold acetonitrile were added to each tube. Samples were then incubated at -80°C for ~45 minutes and centrifuged for five minutes at 4°C and 14,000 rpm. The supernatant was collected and incubated for another 45 minutes. Samples were centrifuged at the same settings and the supernatant was collected. Additional protein precipitation steps (incubation for ~45 minutes and centrifugation for five minutes) were taken if supernatant remained cloudy after the second centrifugation. Once the brain tissue samples were separated from the precipitated proteins, the samples were stored at -80°C until LC-MS/MS analysis.

Protein Concentration Analysis

Brain protein concentrations were measured using a BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). The diluent for standard curve was 10% acetonitrile. A 10 µL aliquot of each brain sample was diluted 1:10 in Milli-Q water before analysis. A 25 µL aliquot of this solution was then loaded into each well. A volume of 200 µL of working reagent was also added to each well. Samples were run in triplicate. Once the plate had been loaded with all standards and samples, it was incubated at 37°C for 30 minutes. The plate was then cooled to room temperature and measured at an absorbance of 526 nm on a SpectraMax® 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Brain neurotransmitter concentrations were normalized to the observed protein concentrations.

LC-MS/MS Analysis for Neurotransmitters

Brain samples were analyzed for 5-HT, 5-HIAA, and GABA using a Shimadzu LC-MS/MS 8030 (Kyoto, Japan) equipped with a communications bus module (CBM-20A), a degasser (DGU-20A$_5$), a liquid chromatograph (LC-20AT), and an auto sampler (SIL-20HT). The mobile phase consisted of 100% methanol and 0.03% formic acid in LC-MS/MS grade water and was run on a gradient (**Table 3.1**). From 2 to 8 minutes, the flow increased from 5 to 95% methanol, from 8 to 11 minutes, the flow remained stable at 95% methanol, and at 11.01 minutes, the flow decreased to 5% methanol to allow for the instrument to recalibrate before the next sample was injected. The injection volume was set at

2 µL. The run time was 11 minutes. Retention times for the analyzed monoamines were 2.1, 5.5, and 9.5 minutes for 5-HIAA, 5-HT, and GABA, respectively.

Table 3.1 LC-MS/MS mobile phase gradient.

Time (min)	% Methanol
2	5
8	95
11	95
11.01	5

Data Analysis

Behavioral data were analyzed using JMP Pro 10 Statistical Discovery software (SAS, Cary, NC, USA). A model was developed that included terms for the fixed effects of drug concentration, time-point, and time-point by concentration interaction; and random effects of exposure number, concentration by exposure number interaction, and tank nested within exposure number and concentration combinations. ANOVA was used to test the significance of the model terms. If model terms were found to be significant, then Fisher's Protected Least Significant Difference test was used to compare the least squares means and determine the nature of the effects.

Brain neurotransmitter data were also analyzed using JMP Pro 10 Statistical Discovery software (SAS, Cary, NC, USA). ANOVA was used to test the significance of the linear relationship between concentration and

neurotransmitter level. Then Dunnett's test was used to compare the means of the control groups to the means of the treatment groups. In addition, behavioral and brain neurotransmitter data were log transformed and ANOVA was used to test the significance of the behavioral data as a function of the brain neurotransmitter data.

CHAPTER 4: RESULTS AND DISCUSSION

Water Quality Measurements

Water quality measurements were averaged (mean ± standard deviation) for all exposures. Measurements included pH, temperature, dissolved oxygen, alkalinity, and hardness. The pH, temperature, dissolved oxygen, alkalinity, and hardness were 8.04 ± 0.31, 23.15°C ± 0.84, 9.16 mg/L ± 0.48, 52.24 mg/L ± 3.66 as $CaCO_3$, and 83.69 mg/L ± 5.24 as $CaCO_3$, respectively.

Diazepam, Buspirone, and Fluoxetine Concentrations

Diazepam

Average diazepam concentrations (mean ± standard deviation) for behavioral exposures were 30.49 ± 8.69, 68.87 ± 14.24, and 136.09 ± 33.22 µg/L. Nominal diazepam concentrations were set at 35, 75, and 150 µg/L. Concentrations remained stable over the course of the exposures. Diazepam concentrations by exposure day are shown in **Figure 4.1**.

Buspirone

Average buspirone concentrations (mean ± standard deviation) for behavioral exposures were 24.90 ± 8.25, 53.43 ± 16.71, and 116.04 ± 39.53 µg/L. Nominal buspirone concentrations were set at 35, 75, and 150 µg/L. Concentrations remained stable over the course of the exposures. Buspirone concentrations by exposure day are shown in **Figure 4.2**.

Fluoxetine

Average fluoxetine concentrations (mean ± standard deviation) for behavioral exposures were 20.93 ± 6.49, 53.72 ± 12.46, and 117.22 ± 36.64 µg/L. Nominal fluoxetine concentrations were set at 35, 75, and 150 µg/L. Concentrations decreased after Day 0, but then remained stable over the remaining days of the exposures. Fluoxetine concentrations by exposure day are shown in **Figure 4.3**.

Copper Concentrations

Average copper concentrations (mean ± standard deviation) for behavioral exposures were 31.86 ± 5.17, 42.95 ± 6.28, and 79.02 ± 11.34 µg/L. Nominal copper concentrations were set at 20, 40, and 80 µg/L. Concentrations remained stable over the course of the exposures. Copper concentrations by exposure day are shown in **Figure 4.4**.

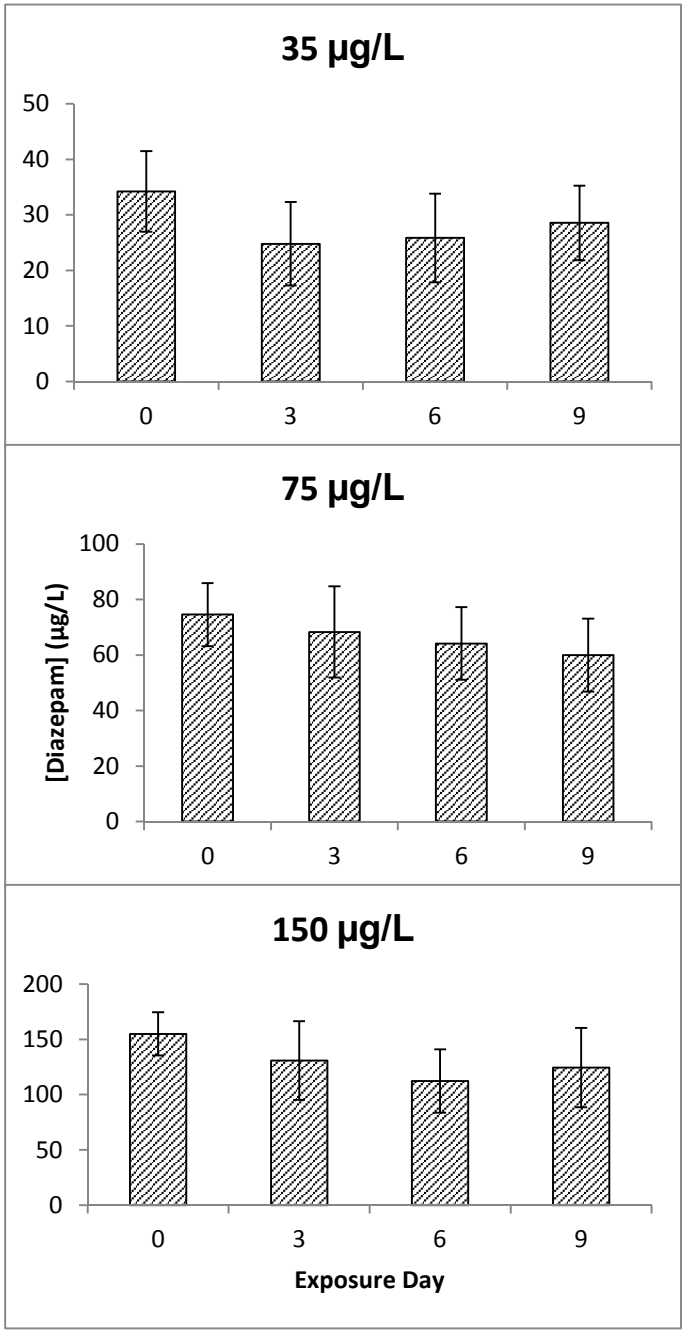

Figure 4.1 Diazepam concentrations throughout the course of the behavioral exposures by exposure day. Nominal concentrations were 35, 75, and 150 µg/L. Error bars represent ±1 standard deviation.

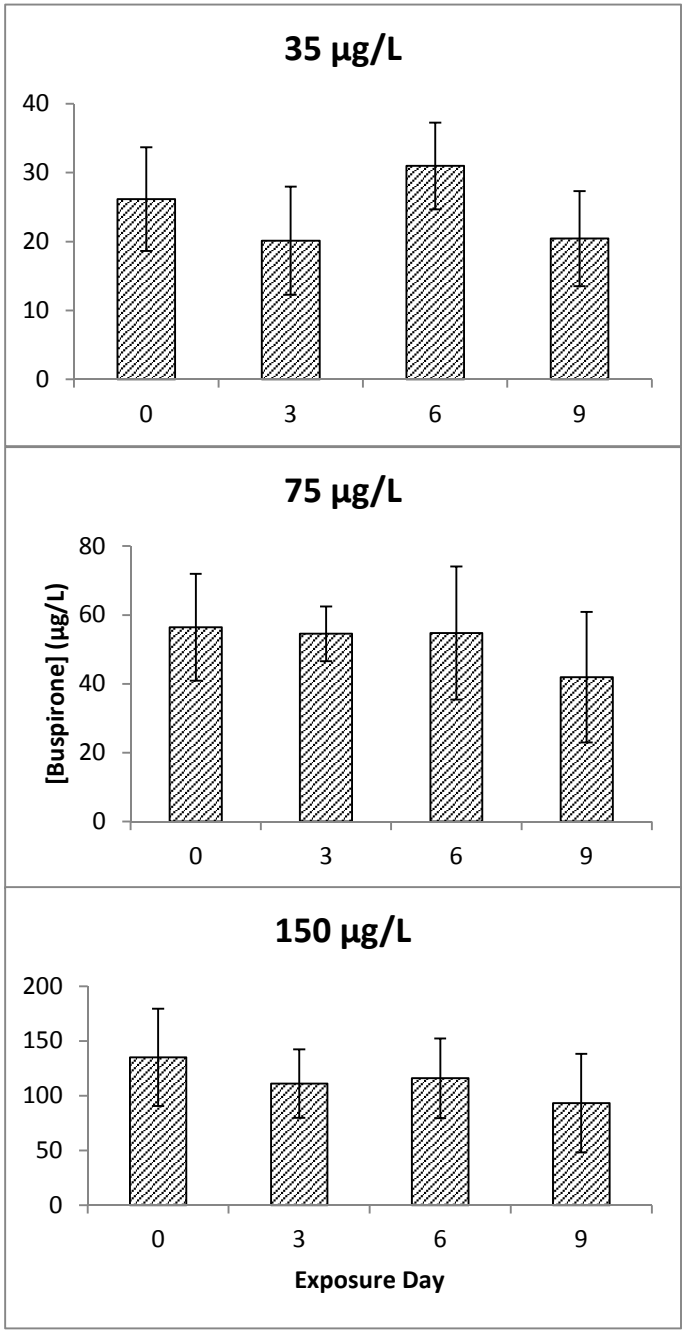

Figure 4.2 Buspirone concentrations throughout the course of the behavioral exposures by exposure day. Nominal concentrations were 35, 75, and 150 µg/L. Error bars represent ±1 standard deviation.

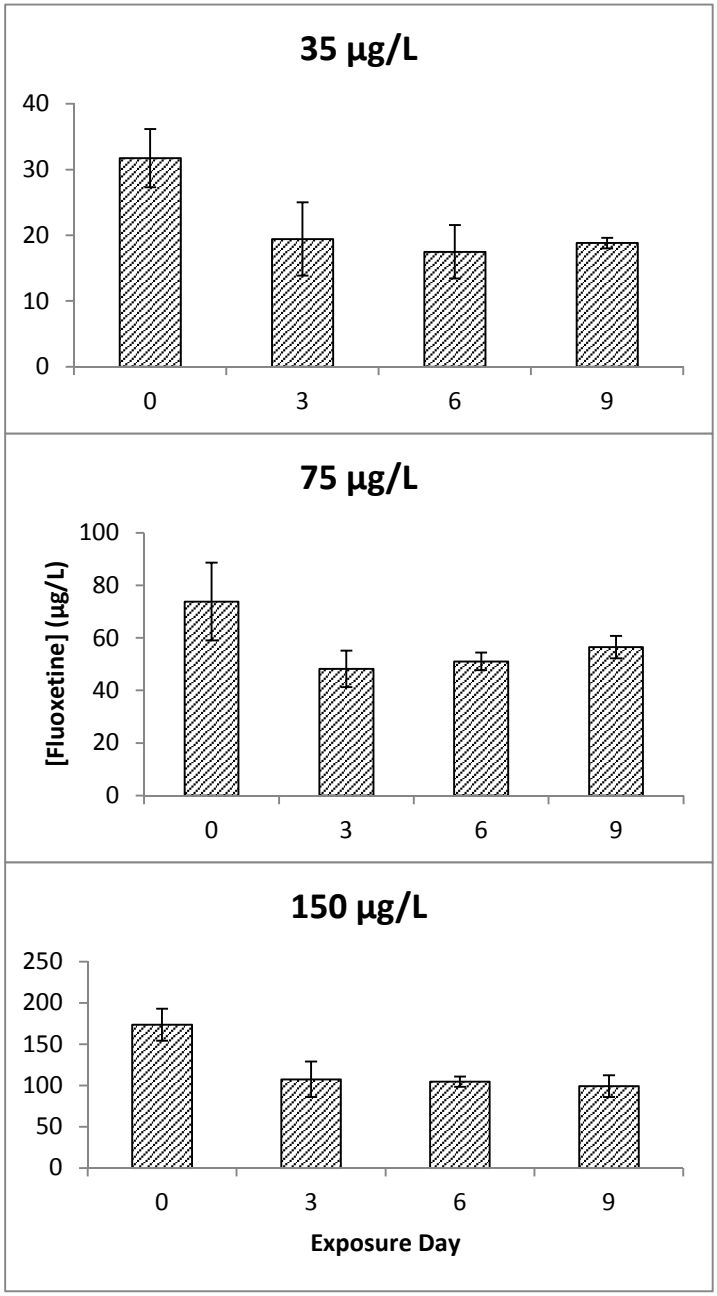

Figure 4.3 Fluoxetine concentrations throughout the course of the behavioral exposures by exposure day. Nominal concentrations were 35, 75, and 150 µg/L. Error bars represent ±1 standard deviation.

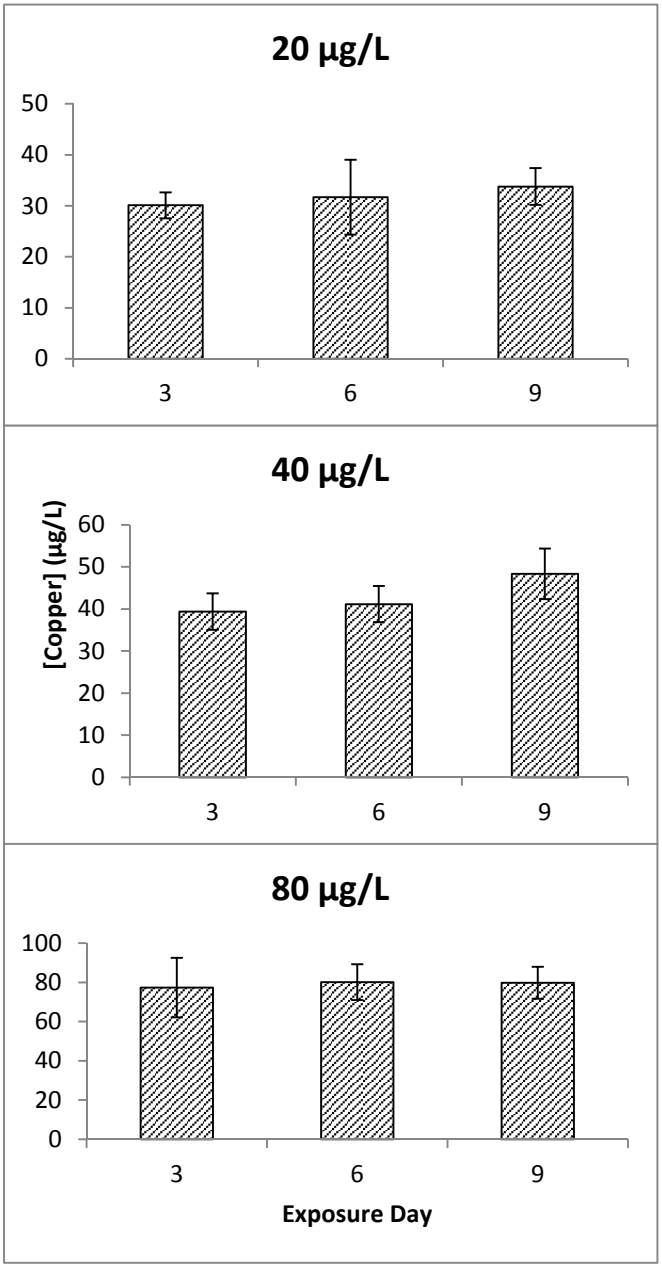

Figure 4.4 Copper concentrations throughout the course of the behavioral exposures by exposure day. Nominal concentrations were 20, 40, and 80 µg/L. Error bars represent ±1 standard deviation.

Qualitative Behavioral Observations

In addition to the two quantitative endpoints discussed below, qualitative observations concerning minnow behavior were also made. During exposures, specifically long-term exposures, minnows seemed less anxious during water changes and re-spikes. Minnows did not flee to the dark side when these events were occurring indicating an anxiolytic state. In addition, "freezing" behavior was observed. Freezing is defined as the complete cessation of movement where only the eyes and gill opercula may move. This behavior has been associated with fear and anxiety-inducing stimuli[64]. Of the fish that displayed freezing behavior, most were either control fish or those exposed to the lowest drug concentration which supports the hypothesis that exposure to anxiolytic drugs reduces anxiety-like behaviors in fish.

Ethanol: Behavioral and Brain Chemistry

After acute exposure to ethanol (positive control, see **Ethanol Exposures**, pg. 30) there were no significant differences among concentrations in number of entries into the light side. There was a significant difference among time-points, however. Number of entries at the 0.5 hr time-point was significantly larger than the number of entries at the 24 hr time-point (**Fig. 4.5**). While the variability among replicates reduced statistical power, the dose-related increase in entries into the light side of the tank at the 0.5 hr time-point after treatment is obvious. The same trend existed for time spent in the light side of the tank at 0.5 hr after

treatment (**Fig. 4.6**). Both of these trends disappear at longer exposure times. These results suggest that ethanol produces an anxiolytic effect in minnows at the onset of exposure, but as time goes on, it has a depressive effect which is not surprising since ethanol is a known sedative. Moreover, as ethanol was the positive control for this assay, I expected an anxiolytic effect.

After acute ethanol exposure, there were significant decreases in all three neurotransmitters measured (**Fig. 4.7**). A strong negative correlation was found between ethanol concentration and serotonin and 5-HIAA levels (R^2=0.8602 and 0.7054, respectively) although the linear relationships between these neurotransmitters and ethanol concentration were not significant (**Table 4.1**). GABA levels decreased in minnows exposed to lower concentrations of ethanol, but increased in minnows exposed to the highest concentration. This relationship was found to be significant with a p-value of 0.0471. As for 5-HIAA, minnows had significantly decreased levels after exposure to 1% ethanol. Serotonin levels were highest, followed by 5-HIAA and GABA.

Minnow GABA and 5-HIAA levels were found to be significantly lower than the control after long-term exposure to 0.25 and 0.5% ethanol (**Fig. 4.8**). Levels of these neurotransmitters increased slightly in minnows after exposure to 1% ethanol. No significant relationships were found between ethanol concentration and serotonin levels, although a decreasing trend in these levels was observed. Serotonin was found in the highest quantity in minnow brains followed by GABA and 5-HIAA.

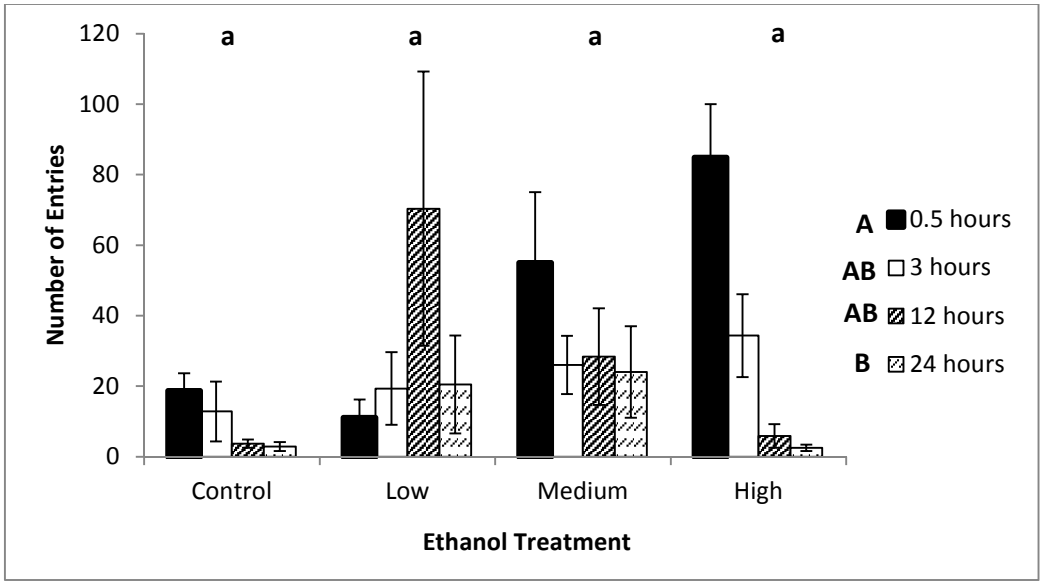

Figure 4.5 Number of entries by minnows to the light side of the tank after acute (1 d) exposure to ethanol. Control = 0%, Low = 0.25%, Medium = 0.5%, High = 1%. Error bars represent ±1 standard error. Capital letters represent statistical differences among time-points. Lowercase letters across the top represent statistical differences among concentrations.

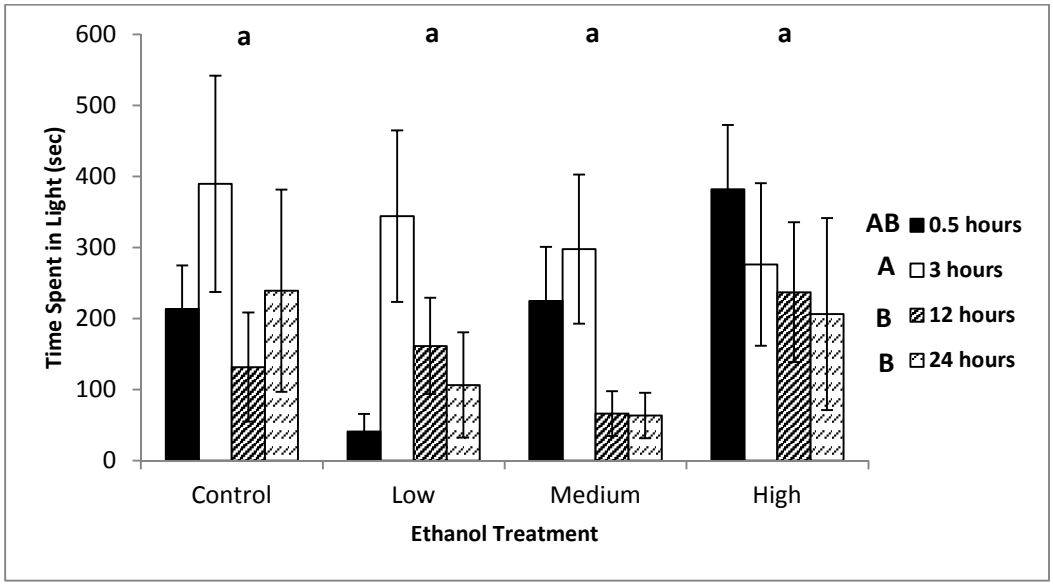

Figure 4.6 Time spent in light by minnows after acute (1 d) exposure to ethanol. Control = 0%, Low = 0.25%, Medium = 0.5%, High = 1%. Error bars represent ±1 standard error. Capital letters represent statistical differences among time-points. Lowercase letters across the top represent statistical differences among concentrations.

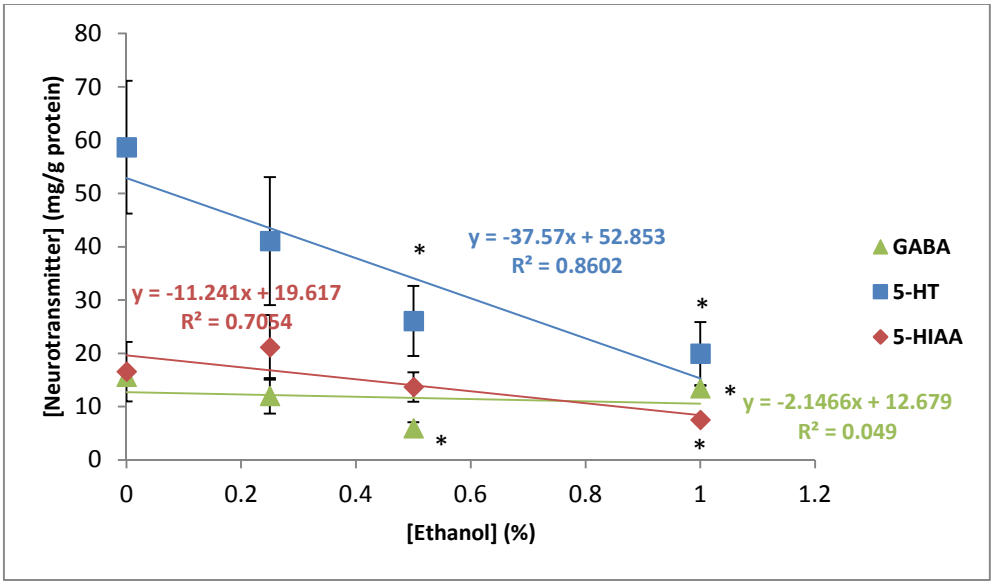

Figure 4.7 Minnow neurotransmitter concentrations after acute (1 d) exposure to 0.25, 0.5, and 1% ethanol. Brains were extracted one day after exposure. Error bars represent ±1 standard error. Asterisks represent significant difference from the control. Equations and correlation coefficients are shown above and below their respective lines. p GABA=0.0471, p 5-HT=0.1385, p 5-HIAA=0.4046.

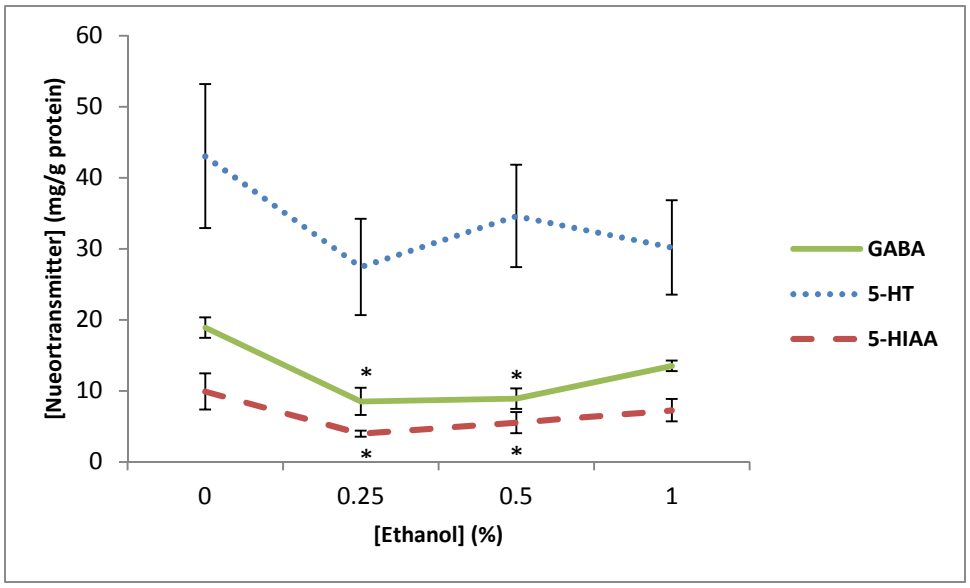

Figure 4.8 Minnow neurotransmitter concentrations after long-term (9 d) exposure to 0.25, 0.5, and 1% ethanol. Brains were extracted one day after exposure. Error bars represent ±1 standard error. Asterisks represent significant difference from the control.

Table 4.1 P-values and R^2 values for neurotransmitter regression lines after acute exposure to ethanol.

Neurotransmitter	P-value	R^2 value
GABA	0.0471	0.049
5-HT	0.1385	0.8602
5-HIAA	0.4046	0.7054

There were no significant differences in either number of entries or time spent on the light side of the tank in the long-term ethanol exposures (**Fig. 4.6**).

Mathur and Guo exposed zebrafish to 0 and 1% ethanol for 8 days (20 min/day) and observed their behavior during a withdrawal period. In contrast to our results, zebrafish exposed to 1% ethanol spent more time in the light compartment relative to control minnows. No effect was found on number of entries to the light compartment[51]. Based on these results, ethanol does not have a long-term effect on fathead minnow locomotion or anxiety behavior.

A previous study observed a concentration effect on both anxiety and locomotion after exposure to ethanol[41]. In that study, zebrafish exposed to 0.5% ethanol had more midline crossings and spent more time in the light compartment relative to control minnows. A second study found that zebrafish exposed to 0.5 and 1% ethanol spent more time in the light compartment compared to control fish. In addition, zebrafish exposed to 1.5% ethanol made more entries to the light compartment, but had decreased time spent in the light compartment[51], which was similar to our results. However, these studies only looked at zebrafish behavior for 15 and 5 minutes each, respectively. In contrast, our study observed minnow behavior for 15 minutes at 4 different time-points after exposure. Further, the second study observed zebrafish during a withdrawal period and not in exposure water, as our study did. We found that minnows were more active and spent more time on the light side early in the exposure and had decreased activity and time spent in the light compartment at later time-points, suggesting an anxiogenic (increase in anxiety) effect of ethanol as the experiment proceeded. Extended exposure to ethanol may have caused

depression in the minnows so that as time passed, they became less active and did not explore the exposure tank. The fact that previous studies only observed fish behavior for a short period of time could explain some of the differences between results.

In addition, a major difference between my study and previous studies is that the latter used zebrafish as a model organism. The light-dark behavioral bioassay was developed using zebrafish[46]. However, I used fathead minnows because they are a ubiquitous species and thus, more relevant in this context. Species differences could be a driving factor causing opposite results in our study as compared to others. Egan *et al.* observed differences in anxiety behaviors between different strains of zebrafish, suggesting a genetic component to anxiety behavior[45]. Thus, this type of behavior may not translate the same way in fathead minnows. In the future, this experiment could be performed using zebrafish to characterize any possible species differences.

As depicted in **Figure 4.7**, there was a trend of dose-dependent decrease in brain serotonin levels in response to increasing ethanol concentration. However, due to high replicate variability this relationship was not found to be statistically significant. Ethanol is a known positive allosteric modulator of the serotonin receptor and has been shown to decrease serotonin levels and affect serotonin metabolism[68,69]. Hence, I expected an effect of ethanol on serotonin levels in the minnow brain. GABA levels also decreased significantly at 0.5%

ethanol. However, minnows exposed to 1% ethanol had increased GABA levels compared to those exposed to 0.5% ethanol. This finding suggests that there is a threshold of ethanol exposure below which GABA levels decrease while above which, there was no observable effect on brain GABA concentrations. Above this threshold, GABA receptors could become saturated, leading to an increase in circulating GABA levels in the brain. In contrast to serotonin, this relationship was found to be significant (p-value=0.0471), suggesting that GABA levels in minnows can be predicted by ethanol exposure.

After long-term exposure to ethanol, minnow GABA and 5-HIAA levels were found to be significantly lower than the control at lower concentrations but increased as ethanol concentration increased thereafter (**Fig. 4.8**). A similar effect was also seen in GABA levels of minnows exposed to acute ethanol. Again, at concentrations higher than 0.5% ethanol, these receptors may become saturated, leading to an increase in these neurotransmitter levels in the brain. No significant relationships were found between ethanol concentration and serotonin levels, although a decreasing trend in these levels was observed which parallels the results from our acute exposure studies. Further, no correlation was observed between either of the behavioral endpoints and any of the measured neurotransmitter levels.

Diazepam: Behavior and Brain Chemistry

After acute exposure to diazepam, there was no statistical significance in number of entries among concentrations or time-point due to high variability among replicates. However, there was an obvious increase in number of entries to the light side by minnows exposed to the two highest concentrations of diazepam (**Fig. 4.9**). This finding suggests an anxiolytic effect of diazepam at higher concentrations. There was a significant difference in number of entries by minnows exposed to 0, 35, and 75 µg/L diazepam long-term (**Fig. 4.10**). A large decrease in entries occurred in minnows exposed to 35 µg/L diazepam, followed by a slight increase in entries at higher concentrations, resulting in a U-shaped response. However, no statistical significance existed among time-points for long-term exposure to diazepam. There were no statistical differences in time spent in the light for either acute or long-term diazepam exposures so these data are not shown.

GABA and 5-HIAA levels decreased following acute diazepam exposure (**Fig. 4.11**).The correlation between diazepam concentration and 5-HIAA levels (R^2=0.8118) was very strong while a weak negative correlation was found between diazepam concentration and GABA levels (R^2=0.2759) (**Table 4.2**). There was also a significant increase in serotonin at the lowest concentration of diazepam followed by a decrease back to control levels.

After long-term exposure to diazepam, both serotonin and 5-HIAA levels significantly decreased at 75 µg/L, but levels subsequently increased at the highest concentration (**Fig. 4.12**). In contrast, there were significantly higher

GABA levels in exposed minnows at lower concentrations with a subsequent decrease at higher concentrations. There were no strong correlations observed between long-term diazepam exposure and neurotransmitter concentrations. For both acute and long-term exposures, the neurotransmitter found at the highest concentrations was 5-HIAA followed by serotonin and GABA.

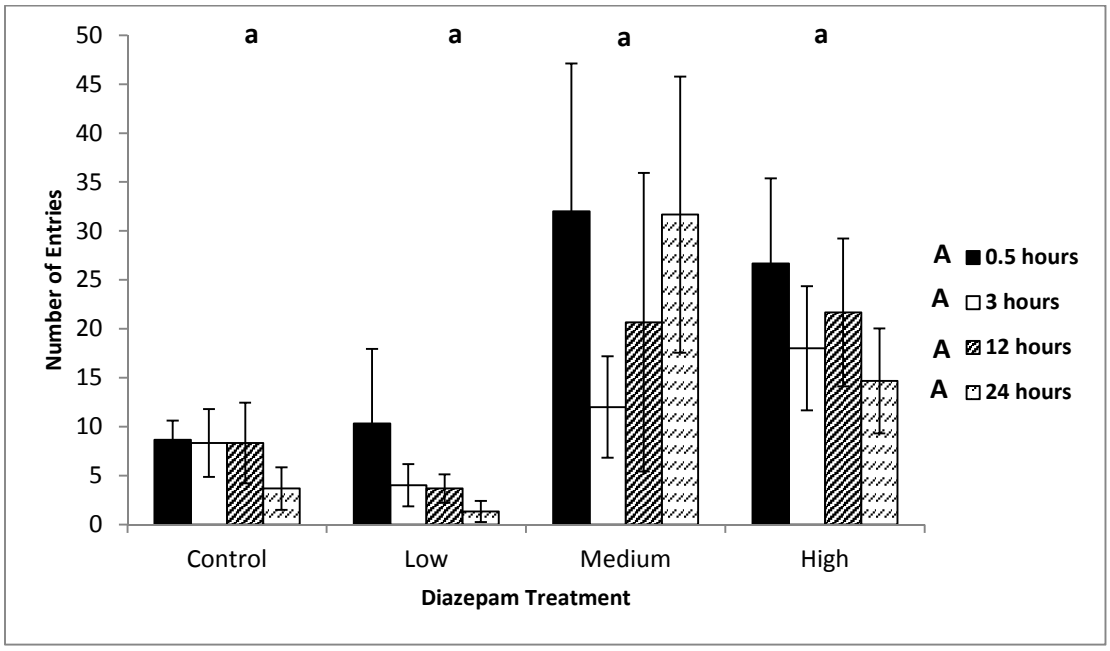

Figure 4.9 Number of entries by minnows to the light side of the tank after acute (1 d) exposure to diazepam. Control = 0 µg/L, Low = 35 µg/L, Medium = 75 µg/L, High = 150 µg/L. Error bars represent ±1 standard error. Capital letters represent statistical differences among time-points. Lowercase letters across the top represent statistical differences among concentrations.

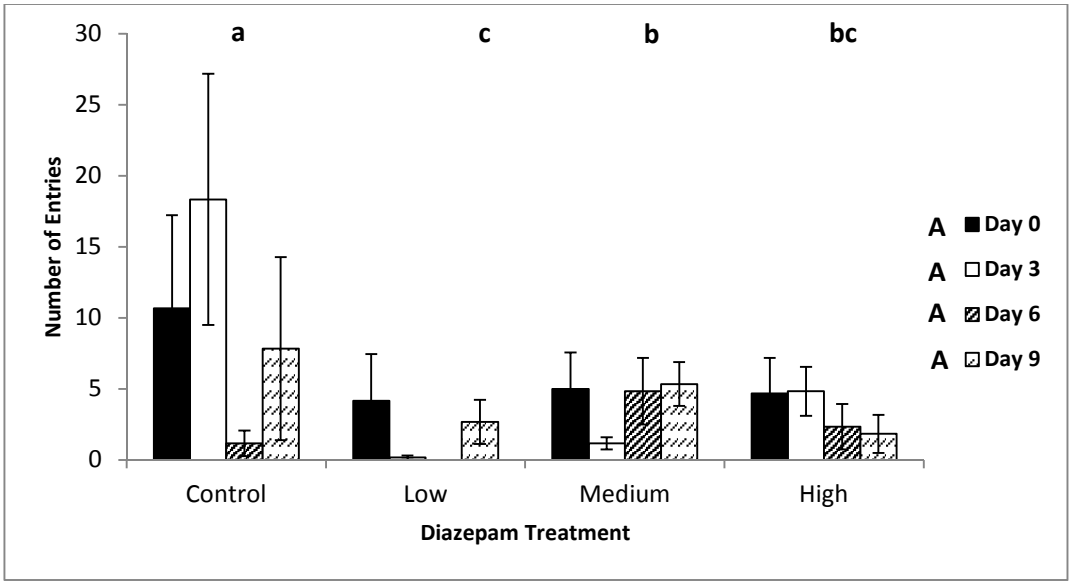

Figure 4.10 Number of entries by minnows to the light side of the tank after long-term (9 d) exposure to diazepam. Control = 0 µg/L, Low = 35 µg/L, Medium = 75 µg/L, High = 150 µg/L. Error bars represent ±1 standard error. Capital letters represent statistical differences among time-points. Lowercase letters across the top represent statistical differences among concentrations.

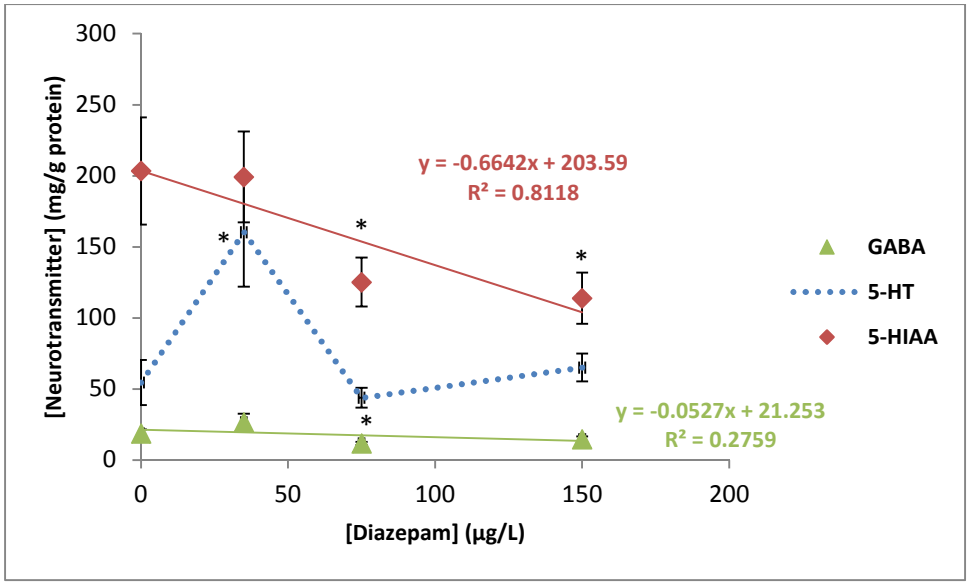

Figure 4.11 Minnow neurotransmitter concentrations after acute (1 d) exposure to 35, 75, and 150 µg/L diazepam. Brains were extracted one day after exposure. Error bars represent ±1 standard error. Asterisks represent significant difference from the control. Equations and correlation coefficients are shown above and next to their respective lines. p GABA=0.0730 and p 5-HIAA=0.1581.

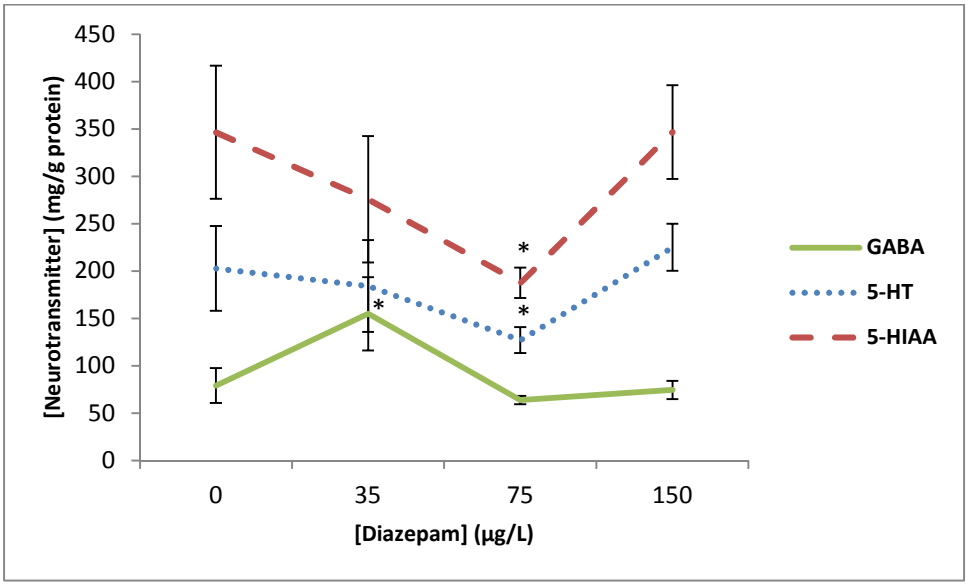

Figure 4.12 Minnow neurotransmitter concentrations after long-term (9 d) exposure to 35, 75, and 150 µg/L diazepam. Brains were extracted ten days after exposure. Error bars represent ±1 standard error. Asterisks represent significant difference from the control.

Table 4.2 P-values and R^2 values for neurotransmitter regression lines after acute exposure to diazepam.

Neurotransmitter	P-value	R^2 value
GABA	0.0730	0.2759
5-HT	Not shown	Not shown
5-HIAA	0.1581	0.8118

After acute exposure to diazepam, there was an obvious increase in number of entries to the light side by minnows at 75 µg/L which continued in the 150 µg/L exposed minnows, suggesting an anxiolytic effect of diazepam on minnow behavior (**Fig. 4.9**). A threshold effect could explain why only at 75 µ/L and above, I found an anxiolytic effect of diazepam on minnow behavior. Previous literature supports this finding of an anxiolytic response in that after exposure to GABA-enhancing drugs like diazepam, rodents and zebrafish displayed anxiolytic behaviors[38].

There was a significant difference in number of entries by minnows exposed to 0, 35, and 75 µg/L diazepam for nine days (**Fig 4.10**). A large decrease in entries occurred in minnows exposed to 35 µg/L diazepam, followed by a slight increase in entries at higher concentrations, suggesting an anxiolytic effect of diazepam exposure on a long-term scale on minnow locomotion at higher concentrations. Similar results were observed in a study that subjected zebrafish to the novel tank diving test after exposure to diazepam. More time

spent on the bottom of the tank is indicative of increased anxiety. Although this study used higher concentrations of diazepam (from 0.625 to 20 mg/L), they observed a U-shaped dose effect on bottom dwelling in zebrafish[66]. Low and high concentrations as well as the middle concentration did not have an anxiolytic effect on bottom dwelling, but moderate concentrations did[66]. Our results parallel their finding in that a U-shaped dose effect was also observed, but with a decrease in entries by control minnows compared to 35 µg/L exposed minnows followed by an increase in entries at higher concentrations.

Acute diazepam exposure resulted in significant decreases in both 5-HIAA and GABA (**Fig. 4.11**). A strong negative correlation was observed between diazepam concentration and 5-HIAA levels (R^2=0.8118) while a weak negative correlation was found between diazepam concentration and GABA levels (R^2=0.2759) (**Table 4.2**). Diazepam is a positive allosteric modulator of GABA type A (GABAA) receptors. Binding of diazepam to these receptors promotes the binding of GABA and GABA-enhancing effects. The enhancement of the GABA-ergic system has been shown to induce anxiolytic effects in several species by inhibiting all other neurotransmitters[38].

After a longer exposure to diazepam, both serotonin and 5-HIAA levels significantly decreased at 75 µg/L, but levels increased in minnows exposed to the highest concentration (**Fig. 4.12**). In contrast, there were significantly higher GABA levels in exposed minnows than in control minnows at lower

concentrations of diazepam, but a subsequent decrease in GABA levels at higher concentrations, although this decrease was not significant. Because of a lack of strong correlations observed between long-term diazepam exposure and neurotransmitter concentrations, I speculate that long-term exposure to diazepam may not have much of an effect on GABA levels in the brain. This relationship provides more insight to the interaction between diazepam and the GABA-ergic system.

My study is the only one in the literature that measured neurotransmitter levels after exposure to diazepam. Thus, it can begin to fill the gap in the area of altered brain chemistry after exposure to anxiolytics. Further, significant correlations were observed between both behavioral endpoints and measured neurotransmitter levels, discussed below.

Buspirone: Behavioral and Brain Chemistry

After exposure to acute buspirone, there was no statistical significance among concentrations for number of entries and no apparent trends among timepoints either (**Fig. 4.13**). After long-term exposure to buspirone, there was no statistical significance among concentrations or time-points for number of entries due to high variability among replicates. However, there was a noticeable trend of more entries to the light side of the tank at lower concentrations compared to higher concentrations (**Fig. 4.14**).

Minnows subjected to an acute exposure to buspirone spent significantly more time on the light side of the tank compared to control minnows (**Fig. 4.15**). This anxiolytic effect of buspirone was immediately apparent. There was also a significant increase in the amount of time that minnows spent on the light side at time-points after 0.5 hours, again suggestive of an anxiolytic effect at higher concentrations.

More definitive results were seen after long-term exposure to buspirone. There was a statistically significant decrease in the time that minnows spent in the light when compared to control minnows, except in minnows exposed to 75 µg/L buspirone at the Day 0 time-point (**Fig. 4.16**). These results suggest a depressive effect of buspirone upon longer exposure.

After acute exposure to buspirone, there was a dose-dependent decrease in serotonin, with an R^2 value of 0.7079 (**Fig. 4.17** and **Table 4.3**). Minnows exposed to the higher concentrations of buspirone had significantly lower levels of serotonin than control minnows. In contrast, 5-HIAA levels increased with a peak at 75 µg/L buspirone followed by a return to control levels at 150 µg/L. Long-term exposure to buspirone yielded a dose-dependent increase in GABA, with an R^2 value of 0.7997 (**Fig. 4.18** and **Table 4.4**). There were no significant differences in measured serotonin or 5-HIAA levels when compared to controls. After both acute and long-term exposure to buspirone, 5-HIAA was the neurotransmitter with the highest levels in the brain followed by serotonin and GABA.

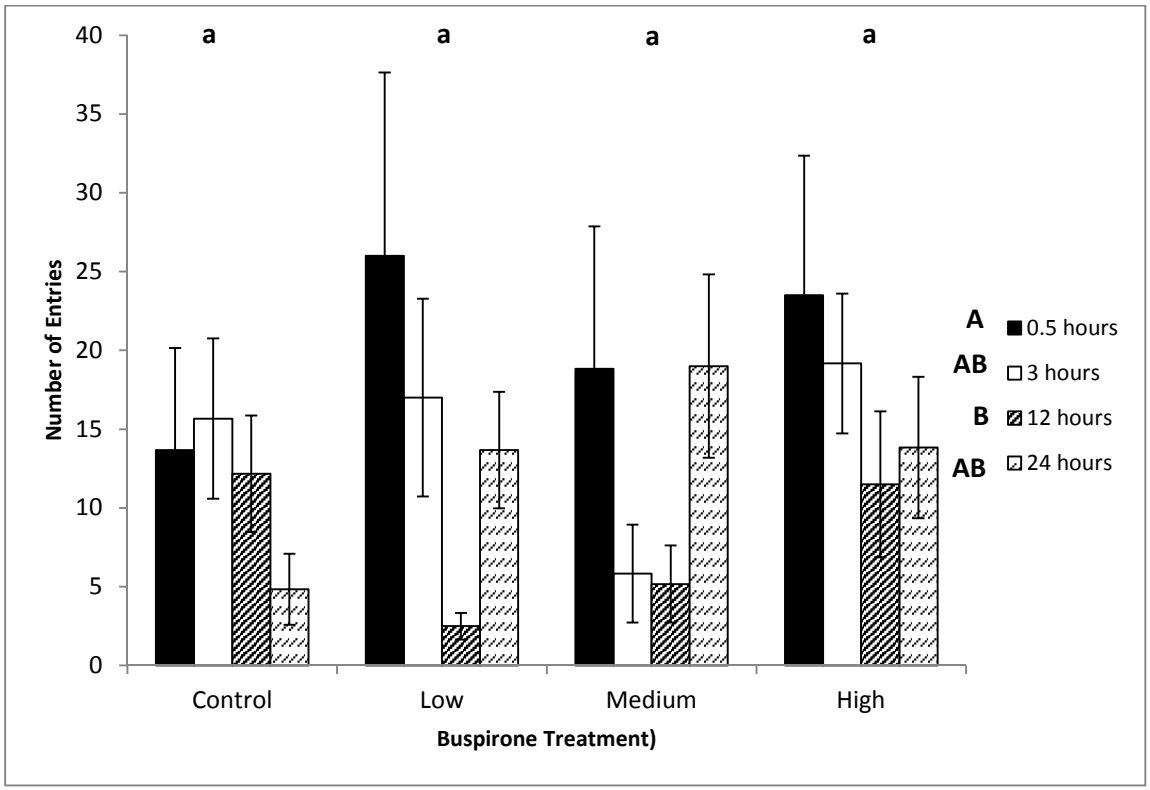

Figure 4.13 Number of entries by minnows to the light side of the tank after acute (1 d) exposure to buspirone. Control = 0 µg/L, Low = 35 µg/L, Medium = 75 µg/L, High = 150 µg/L. Error bars represent ±1 standard error. Capital letters represent statistical differences among time-points. Lowercase letters across the top represent statistical differences among concentrations.

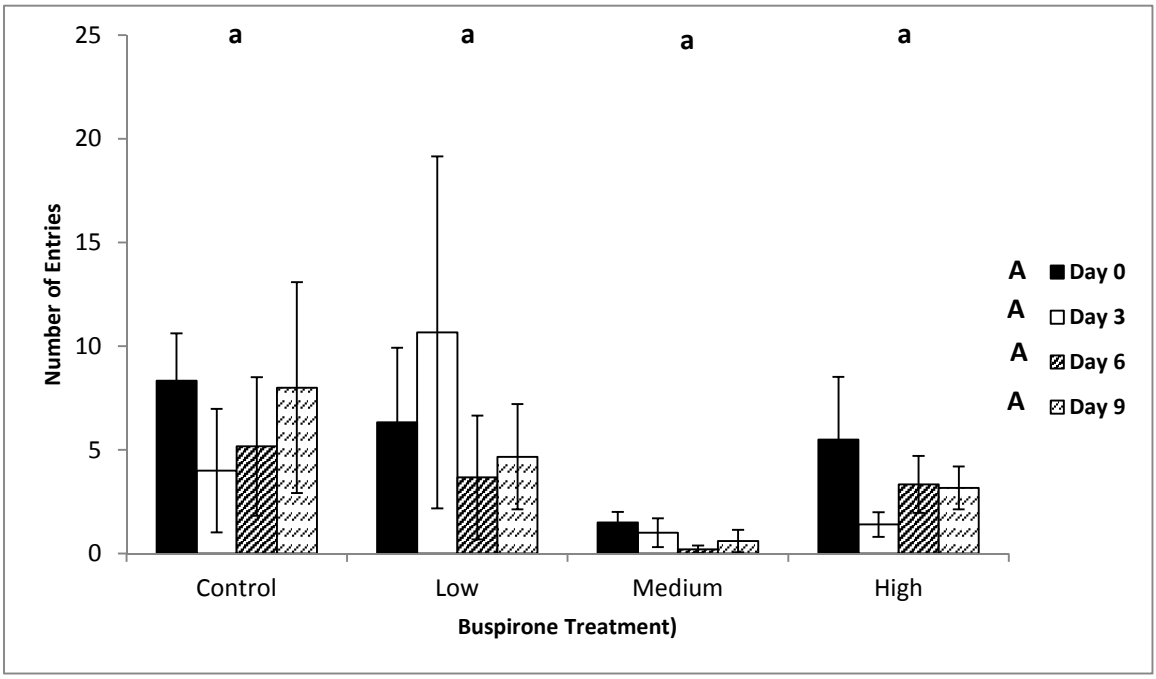

Figure 4.14 Number of entries by minnows to the light side of the tank after long-term (9 d) exposure to buspirone. Control = 0 µg/L, Low = 35 µg/L, Medium = 75 µg/L, High = 150 µg/L. Error bars represent ±1 standard error. Capital letters represent statistical differences among time-points. Lowercase letters across the top represent statistical differences among concentrations.

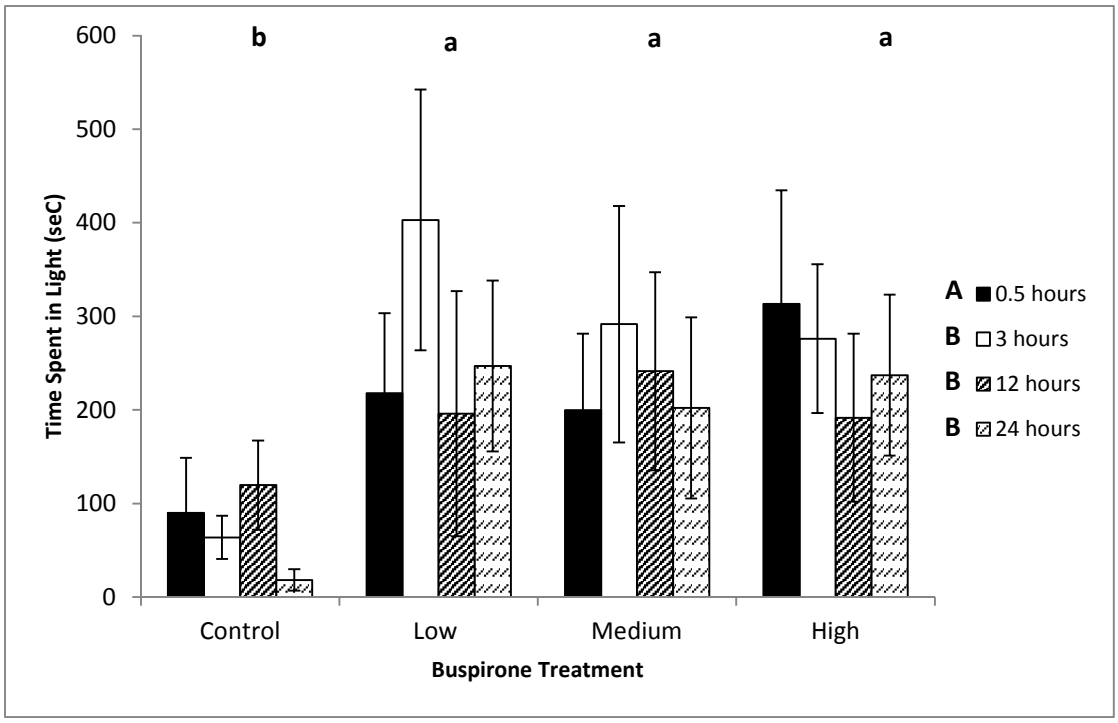

Figure 4.15 Time spent in light by minnows after acute (1 d) exposure to buspirone. Control = 0 µg/L, Low = 35 µg/L, Medium = 75 µg/L, High = 150 µg/L. Error bars represent ±1 standard error. Capital letters represent statistical differences among time-points. Lowercase letters across the top represent statistical differences among concentrations.

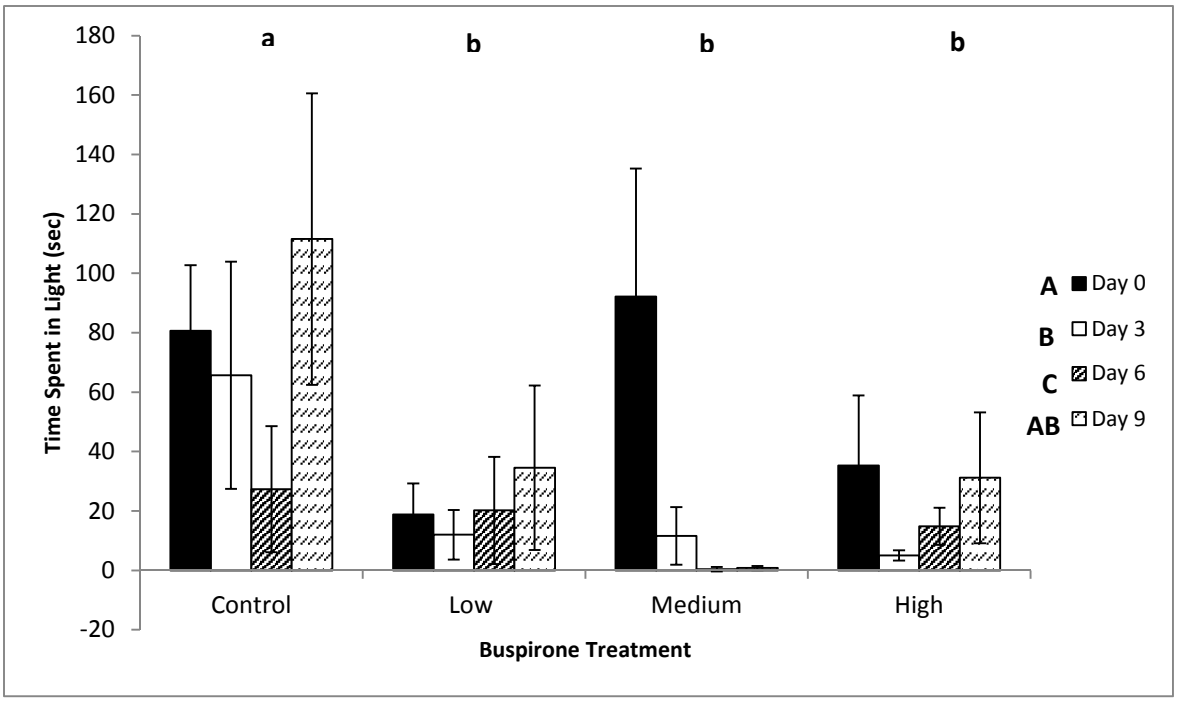

Figure 4.16 Time spent in light by minnows after long-term (9 d) exposure to buspirone. Control = 0 µg/L, Low = 35 µg/L, Medium = 75 µg/L, High = 150 µg/L. Error bars represent ±1 standard error. Capital letters represent statistical differences among time-points. Lowercase letters across the top represent statistical differences among concentrations.

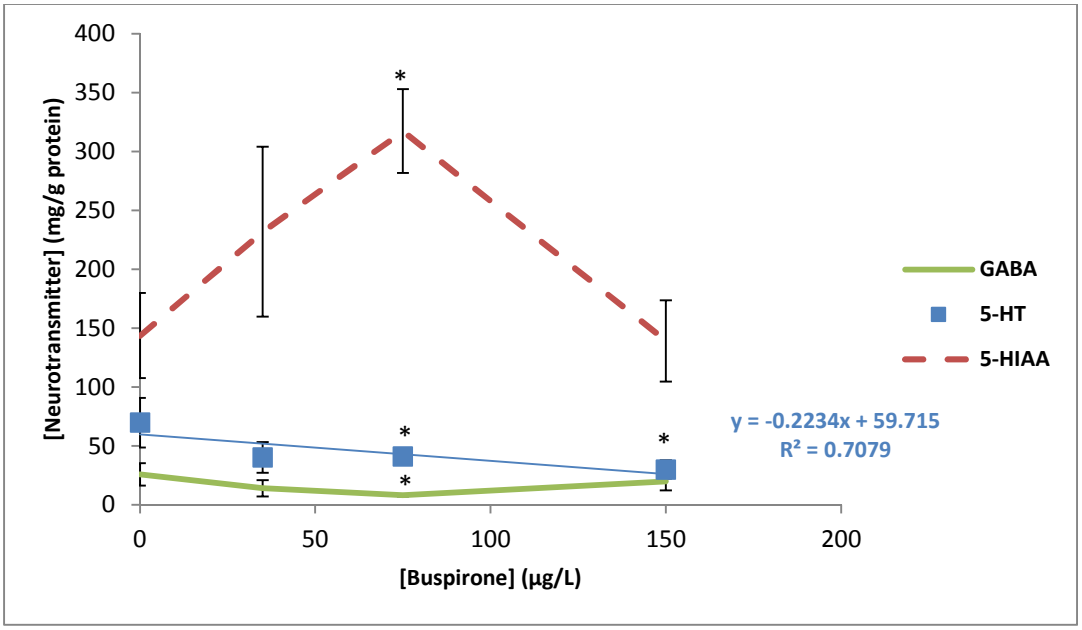

Figure 4.17 Minnow neurotransmitter concentrations after acute (1 d) exposure to 35, 75, and 150 µg/L buspirone. Brains were extracted one day after exposure. Error bars represent ±1 standard error. Asterisks represent significant difference from the control. Equation and correlation coefficient are shown above their respective line. p 5-HT=0.3941.

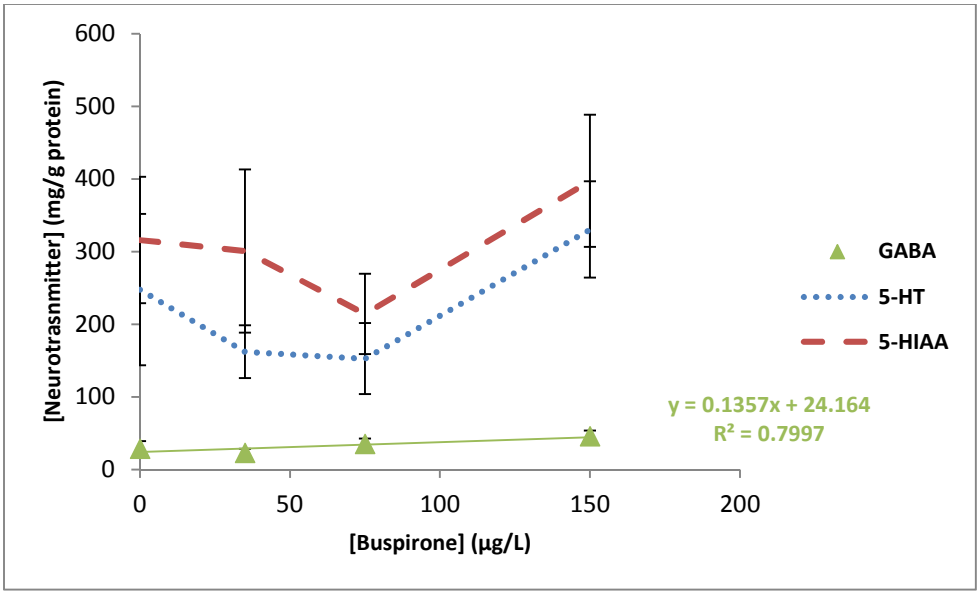

Figure 4.18 Minnow neurotransmitter concentrations after long-term (9 d) exposure to 35, 75, and 150 µg/L buspirone. Brains were extracted ten days after exposure. Error bars represent ±1 standard error. Asterisks represent significant difference from the control. Equation and correlation coefficient are shown next to their respective line. p GABA=0.4098.

Table 4.3 P-values and R^2 values for neurotransmitter regression lines after acute exposure to buspirone.

Neurotransmitter	P-value	R^2 value
GABA	Not shown	Not shown
5-HT	0.3941	0.7079
5-HIAA	Not shown	Not shown

Table 4.4 P-values and R^2 values for neurotransmitter regression lines after long-term exposure to buspirone.

Neurotransmitter	P-value	R^2 value
GABA	0.4098	0.7997
5-HT	Not shown	Not shown
5-HIAA	Not shown	Not shown

After exposure to acute buspirone, I found no statistical significance among concentrations for number of entries (**Fig. 4.13**). These results parallel another study in which zebrafish were injected with buspirone and researchers found no observable effect of buspirone dose on locomotion[41].

After exposure to long-term buspirone, there was no statistical significance among concentrations or time-points for number of entries (**Fig. 4.14**). However, there does seem to be a noticeable trend present. There were more entries at lower concentrations as compared to higher concentrations. Although not significant due to high variability among replicates, the observation suggests that at lower concentrations, long-term exposure to buspirone had an anxiolytic effect on minnows. As concentrations increased, minnows were less active and less willing to explore the tank. Alternatively, minnows could become tolerant to buspirone at higher concentrations, and so are not affected by the drug as much and do not enter the light side as often.

There was a significant and immediate increase in time spent on the light side by exposed minnows as compared to control minnows after acute exposure to buspirone (**Fig. 4.15**). This observation indicates that minnows were less anxious to explore the light side of the tank, suggesting that buspirone had an anxiolytic effect on the minnow's preference for the light side of the tank. Maximino *et al.* found similar results as zebrafish in their experiment exhibited a dose dependent increase in time spent in the light compartment[41]. Connors *et al*. also found similar results: zebrafish exposed to higher concentrations of buspirone spent far more time in the white compartment when compared to minnows exposed to lower concentrations[50]. My study found a significant increase in the amount of time that minnows spent on the light side at time-points after 0.5 hours, again suggestive of an anxiolytic effect of buspirone.

In contrast, after long-term exposure to buspirone, there was a statistically significant decrease in the time that minnows spent in the light when compared to control minnows, except in minnows exposed to 75 µg/L buspirone at the Day 0 time-point, where time spent in light increased again (**Fig. 4.16**). This finding parallels the entry data after long-term exposure to buspirone discussed above. Thus, exposure to long-term buspirone may cause a depressive effect in minnows resulting in less time spent in the light.

As **Figure 4.17** shows, a dose-dependent decrease in serotonin levels after acute exposure to buspirone occurred. An R^2 value of 0.7079 indicated that buspirone exposure is a strong predictor of serotonin levels, although the p-value

of regression (0.3941) was not significant (**Table 4.3**). The result was expected because buspirone is a known serotonin receptor agonist that reduces the synthesis and release of serotonin in the brain[25]. In contrast, a significant increase in 5-HIAA levels was found in minnows after exposure to 75 µg/L buspirone. Because 5-HIAA is a metabolite of serotonin, I would expect an increase in 5-HIAA as serotonin levels decrease.

In contrast to the acute exposure, long-term exposure to buspirone yielded a dose-dependent increase in measured GABA levels although the change was not found to be statistically significant (**Fig. 4.18** and **Table 4.4**). Further, no correlation was observed between either of the behavioral endpoints and any of the measured neurotransmitter levels. My study is the only one in the literature that measured neurotransmitter levels after exposure to buspirone, it serves as a stepping stone for future studies.

Fluoxetine: Behavior and Brain Chemistry

After acute exposure to fluoxetine, minnows entered the light side of the exposure tank more than control minnows. The trend was most obvious at 0.5 and 24 hours after exposure (**Fig. 4.19**). While high variability among replicates reduced statistical power, a dose-related decrease in entries to the light side of the tank is apparent.

After acute exposure to 35 µg/L fluoxetine, minnows spent less time on the light side of the tank than control minnows (**Fig. 4.20**). The time spent in the

light for minnows exposed to 35 µg/L fluoxetine was statistically less than that of all other minnows. At concentrations higher than 35 µg/L fluoxetine, the time spent in the light increased to that of control minnows. Differences in time spent in the light at each time-point were not significant. After long-term exposure to fluoxetine, there were no statistical differences in either behavioral measure, and so those data are not shown.

Acute exposure to 35 µg/L fluoxetine significantly decreased GABA levels in minnows (**Fig. 4.21**). However, these levels were increased after exposure to a higher concentration of fluoxetine. The same positive trend was observed in serotonin and 5-HIAA levels. Minnows exposed to 35 µg/L fluoxetine on a long-term scale had significantly decreased serotonin levels (**Fig. 4.22**). However, these levels significantly increased after exposure to 75 µg/L and remained higher than control minnow serotonin levels after exposure to150 µg/L. Again, the same positive trend was observed with GABA and 5-HIAA levels, results that paralleled what I observed in the acute exposures. Following both acute and long-term exposure to fluoxetine, serotonin was found in the highest quantity followed by 5-HIAA and GABA.

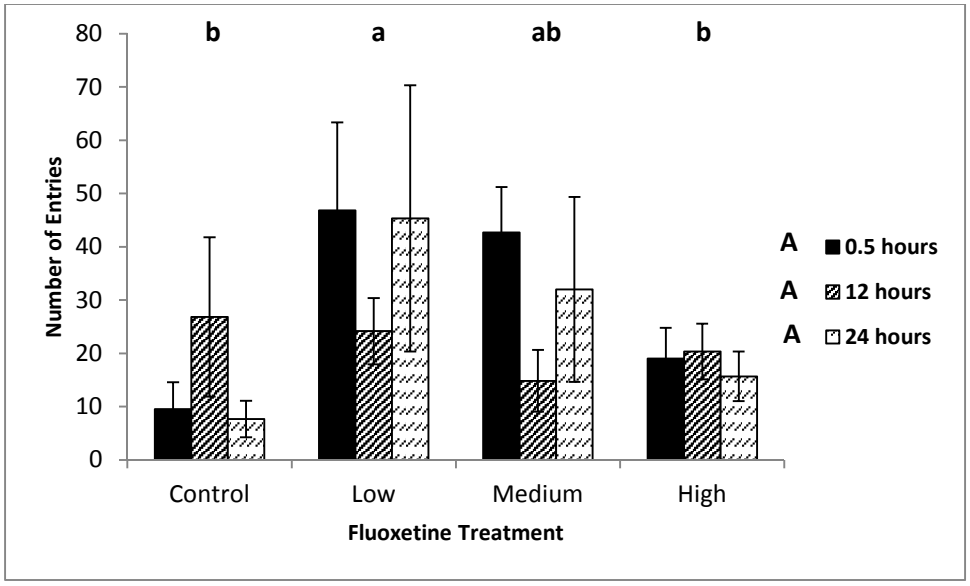

Figure 4.19 Number of entries by minnows to the light side of the tank after acute (1 d) exposure to fluoxetine. Control = 0 µg/L, Low = 35 µg/L, Medium = 75 µg/L, High = 150 µg/L. Error bars represent ±1 standard error. Capital letters represent statistical differences among time-points. Lowercase letters across the top represent statistical differences among concentrations.

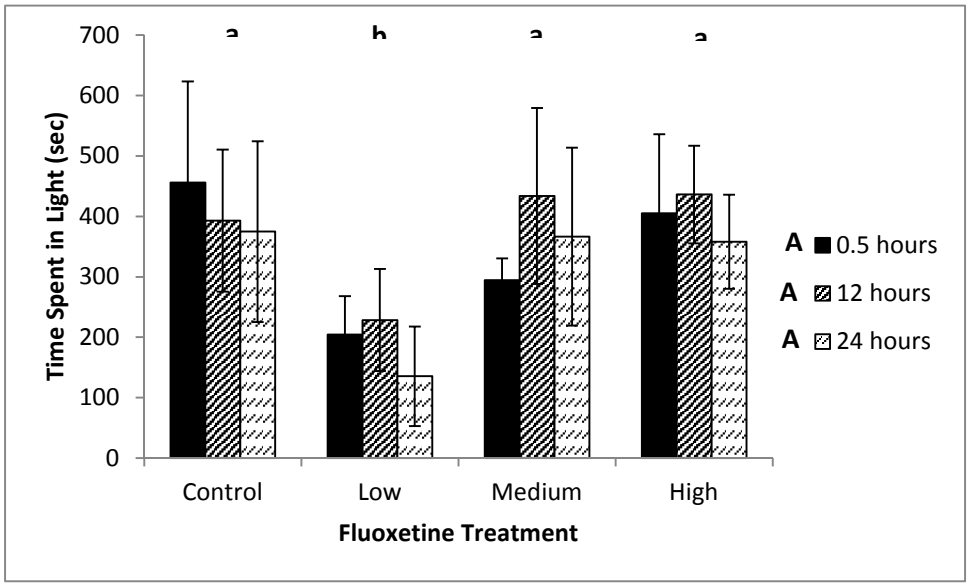

Figure 4.20 Time spent in light by minnows after acute (1 d) exposure to fluoxetine. Control = 0 µg/L, Low = 35 µg/L, Medium = 75 µg/L, High = 150 µg/L. Error bars represent ±1 standard error. Capital letters represent statistical differences among time-points. Lowercase letters across the top represent statistical differences among concentrations.

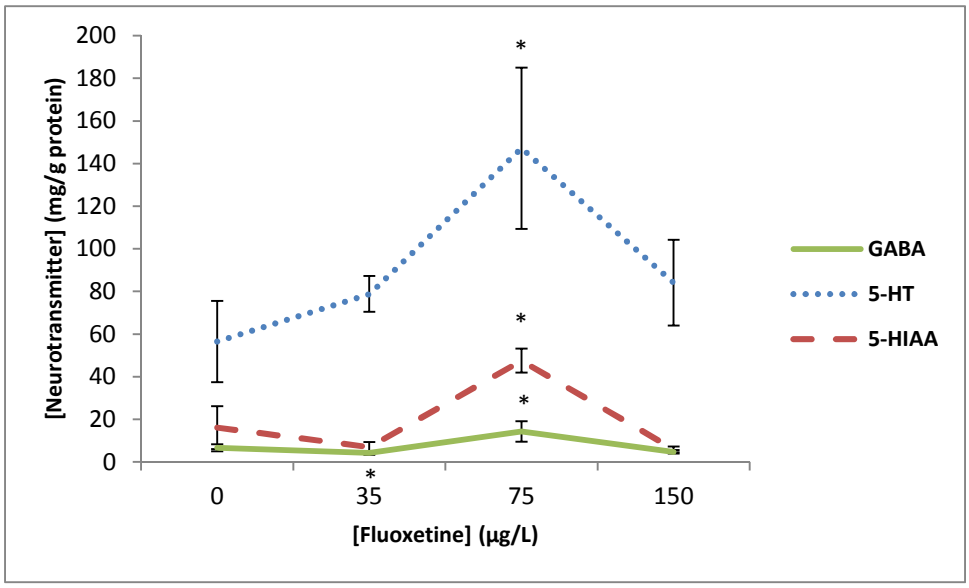

Figure 4.21 Minnow neurotransmitter concentrations after acute (1 d) exposure to 35, 75, and 150 µg/L fluoxetine. Brains were extracted one day after exposure. Error bars represent ±1 standard error. Asterisks represent significant difference from the control.

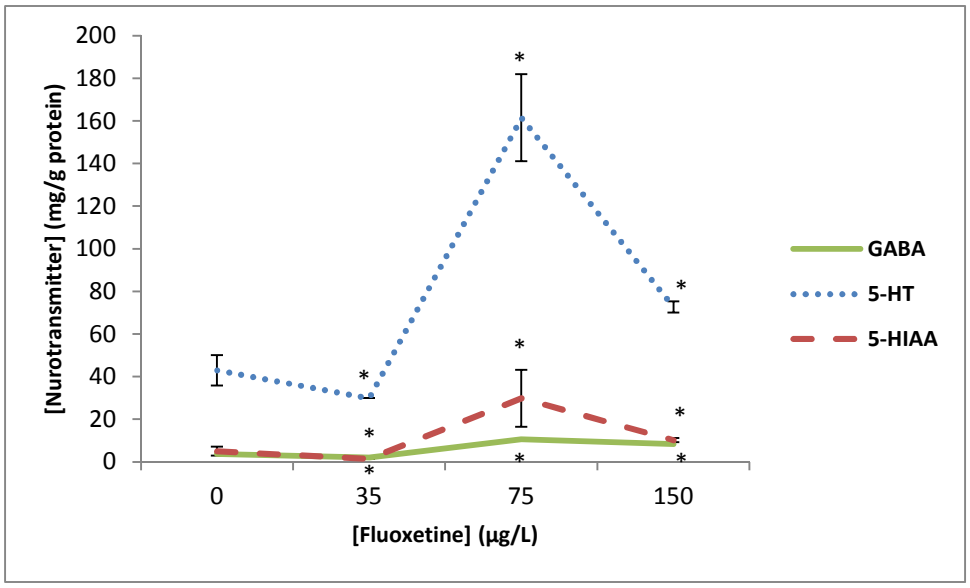

Figure 4.22 Minnow neurotransmitter concentrations after long-term (9 d) exposure to 35, 75, and 150 µg/L fluoxetine. Brains were extracted one day after exposure. Error bars represent ±1 standard error. Asterisks represent significant difference from the control.

Fluoxetine is one of the most studied medications in terms of its effect on organism behavior as evidenced by the plethora of research in the past decade[37,38,41,45,55]. The conclusion from the majority of the research is that exposure to fluoxetine causes anxiolytic effects in fish. One of these studies exposed zebrafish to 100 µg/L fluoxetine for 2 weeks and observed their behavior in the novel tank diving test[45]. Fluoxetine was shown to cause robust anxiolytic effects in the zebrafish, including increased exploration and reduced erratic movements[45]. A second experiment subjected zebrafish to the light-dark scototaxis test after injection with either 5 or 10 mg/kg fluoxetine[41]. Researchers

observed altered locomotion and more time spent in the light compartment by zebrafish at the highest dose after chronic injection with fluoxetine[41]. However, fluoxetine was found to have no effect on zebrafish behavior after 20 minutes of exposure[38].

After acute exposure to fluoxetine, we observed that minnows entered the light side of the exposure tank more than control minnows (**Fig. 4.19**). The trend was most apparent at 0.5 and 24 hours after exposure, although it was not found to be statistically significant due to high variability among replicates. These results parallel previous studies that observed anxiolytic behaviors in zebrafish after fluoxetine treatment. However, at higher concentrations of fluoxetine, a decrease in entries was observed. As **Figure 4.20** depicts, after acute exposure to 35 µg/L fluoxetine, minnows spent significantly less time on the light side of the tank than control minnows. At concentrations higher than 35 µg/L fluoxetine, the time spent in the light increased to that of control minnows.

In contrast with previous studies, I found a decreasing trend in anxiolytic behaviors, specifically minnow entries, after exposure. These differences between my research and previous research could be explained by species differences, differences in route of exposure, and differences in the duration of exposure and observation periods. Previous studies exposed zebrafish to fluoxetine for a shorter period of time than my study as well as observed them for a shorter period of time. A shorter exposure and observation period would explain why I observed an anxiolytic effect at the beginning of the experiment

followed by a decrease in entries over the course of the experiment. In addition, injection of the drug is a faster route of exposure than simple waterborne immersion and thus, the effects of the drug would manifest themselves more rapidly after injection.

I did not find any significant effects of long-term fluoxetine treatment on minnow anxiety behavior although several past studies have documented an anxiolytic effect after two weeks of fluoxetine treatment[45]. Again, the difference could be attributed to species differences as well as differences in route of exposure and duration of exposure/observation periods.

After acute exposure to fluoxetine, neurotransmitters reached their maximum level at 75 µg/L and subsequently decreased to levels similar to those of controls **(Fig. 4.21)**. These results do not parallel previous results from our lab or other studies performed as I observed an increase in all neurotransmitter concentrations when compared to the controls. After exposure to the other compounds in my study, there were observable decreases in brain neurotransmitter concentrations. A previous study also reported decreased serotonin levels in hybrid striped bass after exposure to the same concentrations of fluoxetine for six days[37]. The most obvious difference between the conflicting results is the species of fish used. It may be that fathead minnows respond differently to fluoxetine exposure compared to larger fish species such as the hybrid striped bass.

As depicted in **Figure 4.22**, minnows exposed to 35 µg/L fluoxetine on a long-term scale had significantly decreased serotonin levels. However, these levels significantly increased after exposure to higher concentrations of fluoxetine and remained higher than control levels at the highest concentration. The same trend was observed with GABA and 5-HIAA levels and also, paralleled what I observed after acute exposure to fluoxetine. After chronic treatment with SSRIs, antidepressant effects such as a rise in serotonin levels have been reported[71,72] which could explain my observations of increased neurotransmitter levels in minnow brains as minnows were exposed to fluoxetine for nine days, much longer than previous literature reports. Moreover, despite increases in all neurotransmitters, serotonin levels were most affected by fluoxetine treatment which was expected as fluoxetine is a known SSRI. Further, no correlation was observed between either of the behavioral endpoints and any of the measured neurotransmitter levels.

Copper: Behavioral and Brain Chemistry

After acute exposure to copper, there was an increasing trend in entries to the light side of the tank at and above 40 µg/L (**Fig. 4.23**). However, due to high variability among replicates, the trend was not statistically significant. Minnows exposed to these concentrations entered the light side more than control minnows and those minnows exposed to the lowest concentration of copper.

Moreover, in regards to time spent in the light, there was a significant difference among time-points, specifically 12 and 24 hours after exposure (**Fig. 4.24**).

There were no statistical differences in the long-term exposures in either number of entries or time spent in the light. Thus, I conclude that copper does not have a long-term effect on fathead minnow anxiety behavior. My study, as far as I know, is the only study to investigate the effects of copper exposure, both on an acute and long-term scale, on anxiety behavior in fathead minnows.

A strong negative correlation was found between copper concentration and all neurotransmitter levels after an acute exposure (**Fig. 4.25**). 5-HIAA levels were significantly decreased at 20 µ/L, GABA levels at 20 µg/L, and serotonin levels at the highest concentration, which was 80 µg/L. The neurotransmitter found in the highest quantity was 5-HIAA followed by serotonin and GABA. After long-term exposure to copper, however, only GABA and serotonin levels were significantly decreased when compared to controls (**Fig. 4.26**). Moreover, a strong negative correlation was found between copper concentration and serotonin and GABA concentrations (R^2=0.7257 and 0.9645, respectively) (**Table 4.6**). The relationship between serotonin levels and copper concentration was found to be statistically significant (p=0.0311).

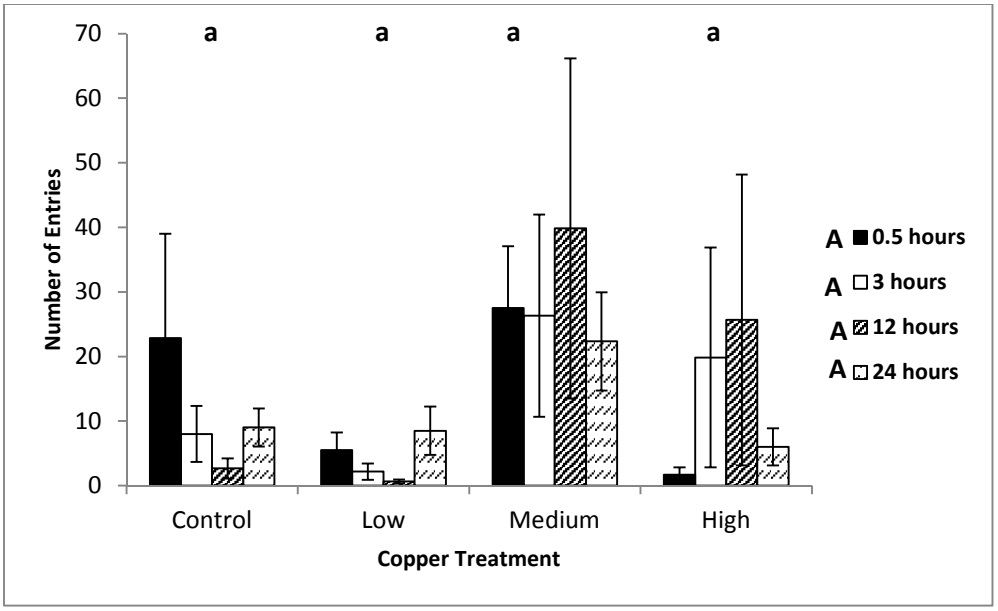

Figure 4.23 Number of entries by minnows to the light side of the tank after acute (1 d) exposure to copper. Control = 0 µg/L, Low = 20 µg/L, Medium = 40 µg/L, High = 80 µg/L. Error bars represent ±1 standard error. Capital letters represent statistical differences among time-points. Lowercase letters across the top represent statistical differences among concentrations.

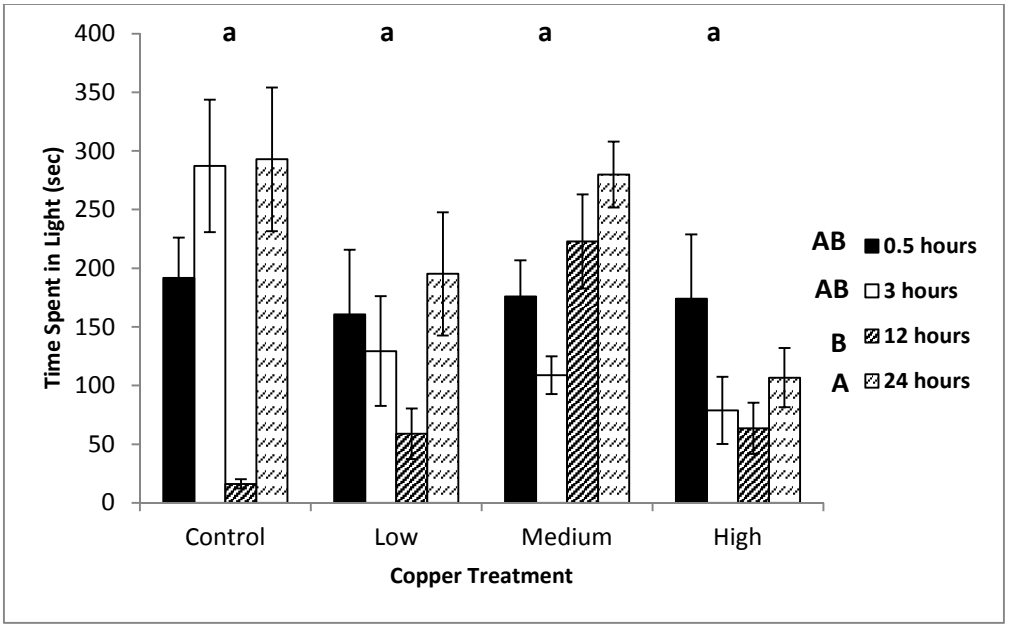

Figure 4.24 Time spent in light by minnows after acute (1 d) exposure to copper. Control = 0 µg/L, Low = 20 µg/L, Medium = 40 µg/L, High = 80 µg/L. Error bars represent ±1 standard error. Capital letters represent statistical differences among time-points. Lowercase letters across the top represent statistical differences among concentrations.

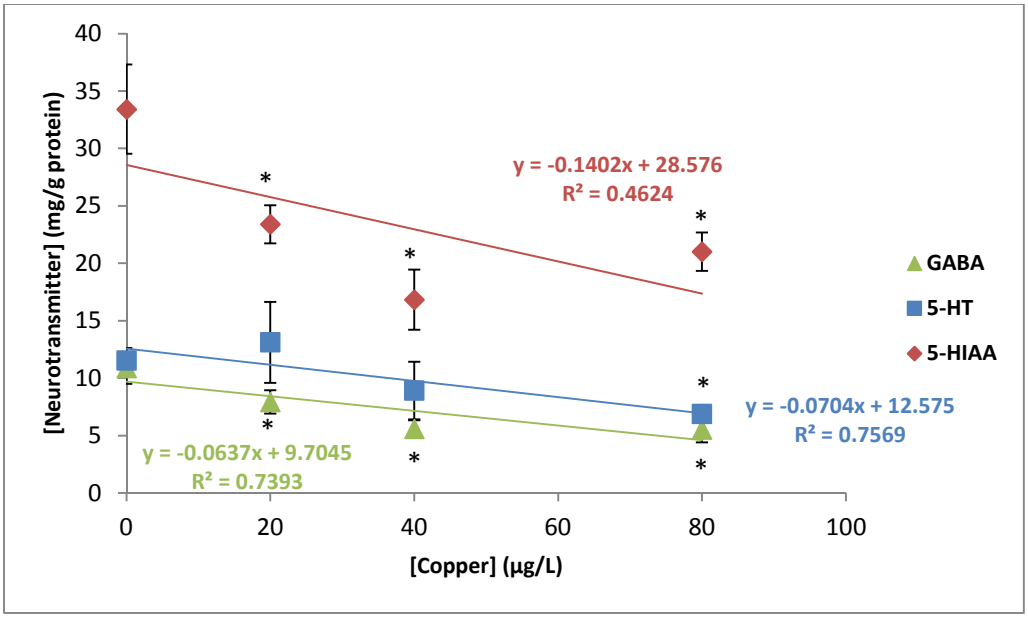

Figure 4.25 Minnow neurotransmitter concentrations after acute (1 d) exposure to 20, 40, and 80 µg/L copper. Brains were extracted one day after exposure. Error bars represent ±1 standard error. Asterisks represent significant difference from the control. Equations and correlation coefficients are shown above and below their respective lines. p GABA=0.1111, p 5-HT=0.5598, p 5-HIAA=0.1088.

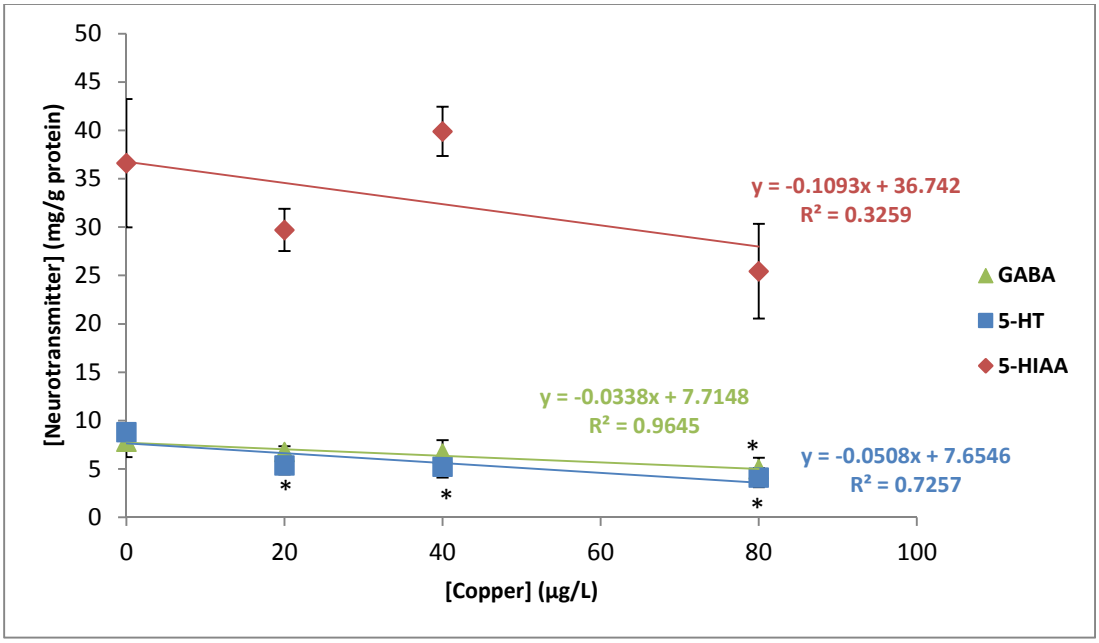

Figure 4.26 Minnow neurotransmitter concentrations after long-term (9 d) exposure to 20, 40, and 80 µ/L copper. Brains were extracted ten days after exposure. Error bars represent ±1 standard error. Asterisks represent significant difference from the control. Equations and correlation coefficients are shown above and next to their respective lines. p GABA=0.5390, p 5-HT=0.0311, p 5-HIAA=0.1921.

Table 4.5 P-values and R^2 values for neurotransmitter regression lines after acute exposure to copper.

Neurotransmitter	P-value	R^2 value
GABA	0.1111	0.7393
5-HT	0.5598	0.7569
5-HIAA	0.1088	0.4624

Table 4.6 P-values and R^2 values for neurotransmitter regression lines after long-term exposure to copper.

Neurotransmitter	P-value	R^2 value
GABA	0.5390	0.9645
5-HT	0.0311	0.7257
5-HIAA	0.1921	0.3259

After acute exposure to copper, there was an increasing trend in entries to the light side of the tank in minnows exposed to higher concentrations of copper (**Fig. 4.23**). Minnows exposed to lower concentrations of copper entered the light side of the tank less often than those minnows exposed to higher concentrations. A proposed mode of action for copper is interference with the olfactory sensory neurons that are embedded in fish epithelial tissue[32]. This interference can cause alterations in predator avoidance behavior of prey so that prey fish cannot sense that a predator is near and has been shown to alter fish preference for the light or dark side in behavioral testing[33]. If the olfactory sense is affected negatively by copper, fish may behave differently and enter the environment that, innately, they do not prefer, possibly making them susceptible to a predator.

In addition, my results support the conclusion of a threshold of copper at 40 µg/L such that until this critical concentration is reached, fish prefer the dark side of the tank. Moreover, in regards to time spent in the light, there was a significant difference among time-points, specifically 12 and 24 hours after

exposure. Exposed minnows spent more time on the light side of the tank after the 24 hr time-point than the 12 hr time-point (**Fig. 4.24**), suggesting a threshold of exposure time as well.

As depicted in **Figure 4.25**, a strong negative correlation was found between copper concentration and neurotransmitter levels. 5-HIAA levels were significantly decreased at 20 µ/L, GABA levels at 20 µg/L, and serotonin levels at the highest concentration, 80 µg/L, with R^2 values of 0.4624, 0.7393, and 0.7569 for 5-HIAA, GABA, and serotonin, respectively (**Table 4.5**). Thus it seems that copper concentration would be a good predictor of brain neurotransmitter levels. These results were expected because it has been proposed that copper affects both the HPA access and serotonin metabolism in the brain by causing cross-linking between molecules[34,70]. These decreased neurotransmitter levels may cause differences in fish behavior, leaving them more susceptible to predation.

After long-term exposure to copper, significant decreases were seen in serotonin levels of exposed minnows (**Fig. 4.26**). Moreover, GABA levels in exposed minnows also decreased relative to controls. A strong negative correlation was observed between long-term exposure to copper and neurotransmitter concentrations, specifically serotonin and GABA (R^2=0.7257 and 0.9645, respectively) (**Table 4.6**). These correlation coefficients suggest that copper concentration is a good predictor of serotonin and GABA levels in the brain on a long-term scale. Additionally, I found a significant linear relationship

between copper concentration and serotonin levels (p=0.0311) which adds to my conclusion that exposure to copper on a long-term scale decreases serotonin levels in minnow brains.

My long-term results parallel a previous study that exposed juvenile carp to sub-lethal levels of copper for one week[28]. Researchers observed a dose-dependent decrease in serotonin levels in three different parts of the brain: the hypothalamus, the tencephalon, and the brain stem. In addition, they observed no significant changes in 5-HIAA[28], also parallel to my study, where the R^2 value for 5-HIAA levels versus copper concentration was 0.2119. Thus, I conclude that long-term copper concentration does not affect 5-HIAA levels in the brain, and is not a good predictor of this endpoint. Because 5-HIAA levels were found to be decreased after acute exposure to copper, but fluctuated after long-term exposure, I speculate that these receptors become tolerant, and thus, copper does not have as much of an effect in the long-term. Further, no correlation was observed between either of the behavioral endpoints and any of the measured neurotransmitter levels.

Linking Behavior with Brain Chemistry

When plotted on a log-log plot, there was a strong negative relationship between both number of entries into the light side and the time spent on the light side of the tank and measured GABA concentrations after acute diazepam exposure. Variability among replicates resulted in p-values that were not

considered statistically significant (p=0.0635 and 0.0514, respectively). Nevertheless, an obvious relationship is present. A weak, negative correlation was observed for both of these relationships with R^2 values of 0.148 and 0.1617, respectively.

When plotted on a log-log plot, there was a significant relationship between both the number of entries into the light side and the time spent on the light side of the tank and measured 5-HIAA concentrations after acute diazepam exposure with p values of 0.0274 and 0.0148, respectively (**Figs. 4.29** and **4.30**). A weak, negative correlation was observed for both of these relationships (R^2=0.2023 and 0.2412, respectively).

A significant relationship was observed between both the log number of entries and log time spent in the light and log [serotonin] after long-term diazepam exposure (**Figs. 4.31** and **4.32**). The p-values were 0.0255 and 0.0409, respectively. Both of these positive relationships were found to be weakly correlated (R^2=0.2159 and 0.1843, respectively).

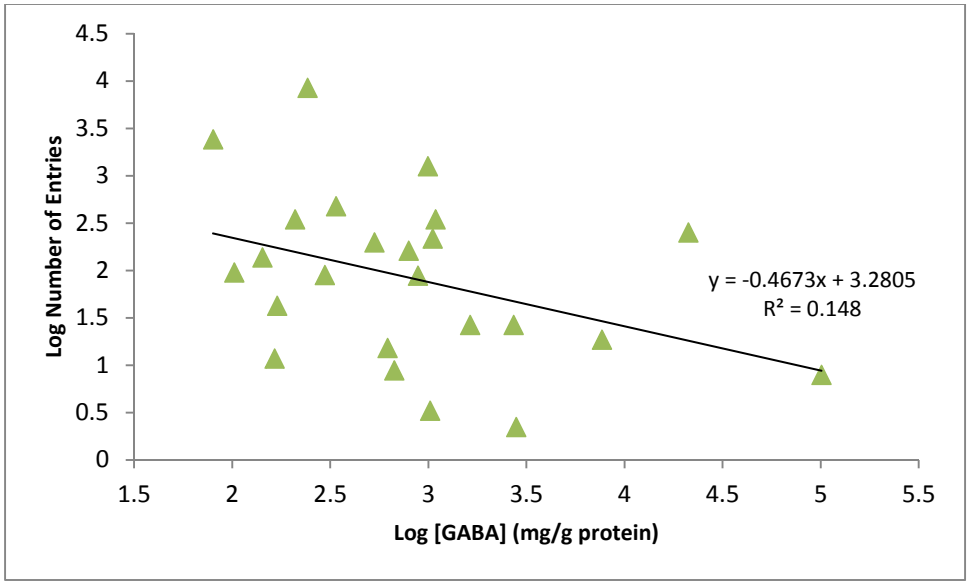

Figure 4.27 Double log plot of fish behavior (number of entries into the light side of the tank) as a function of measured GABA concentrations after acute (1 d) diazepam exposure. The equation and correlation coefficient are depicted. p=0.0635.

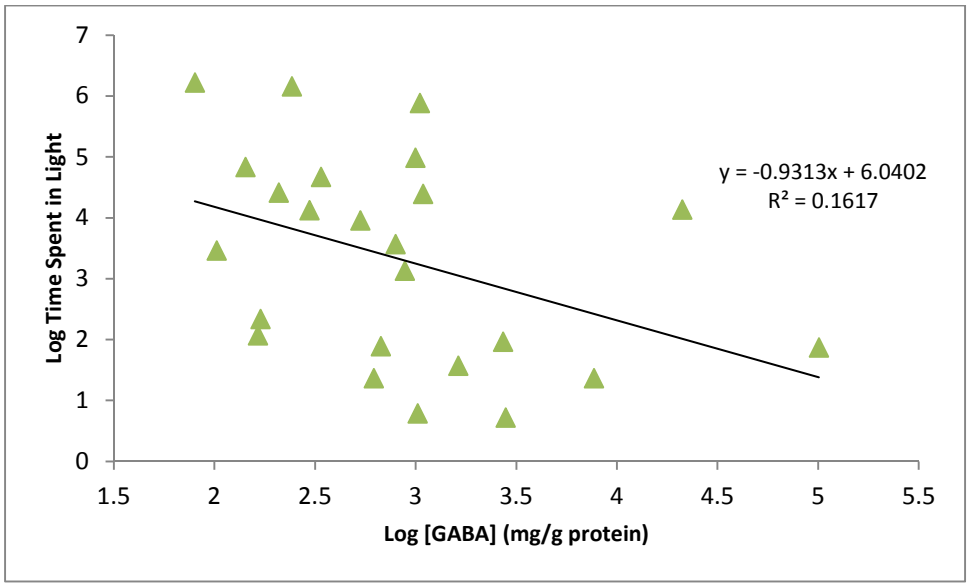

Figure 4.28 Double log plot of fish behavior (time spent on the light side of the tank) as a function of measured GABA concentrations after acute (1 d) diazepam exposure. The equation and correlation coefficient are depicted. p=0.0514.

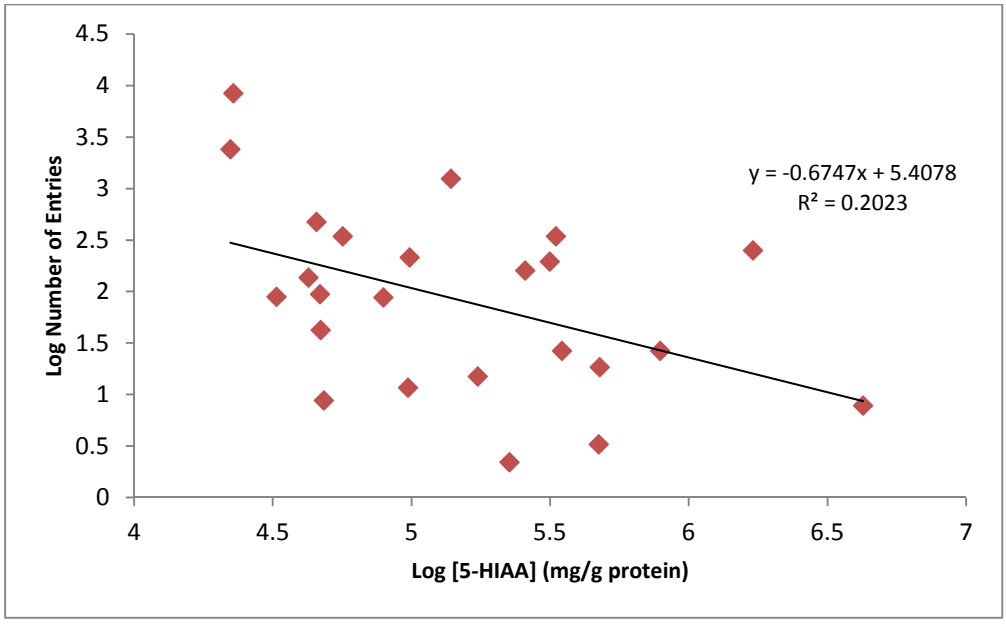

Figure 4.29 Double log plot of fish behavior (number of entries into the light side of the tank) as a function of measured 5-HIAA concentrations after acute (1 d) diazepam exposure. The equation and correlation coefficient are depicted. p=0.0274.

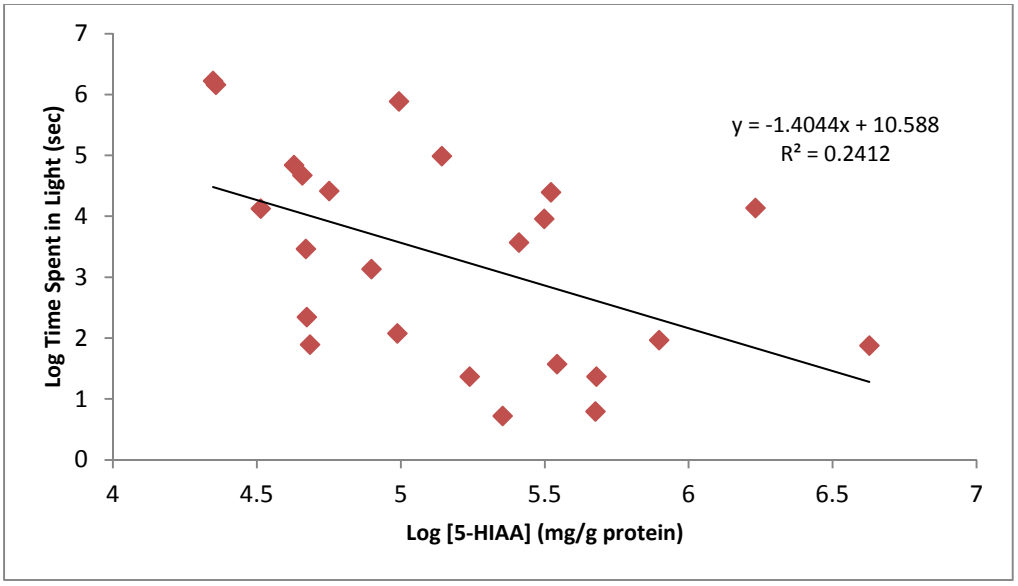

Figure 4.30 Double log plot of fish behavior (time spent on the light side of the tank) as a function of measured 5-HIAA concentrations after acute (1 d) diazepam exposure. The equation and correlation coefficient are depicted. p=0.0148.

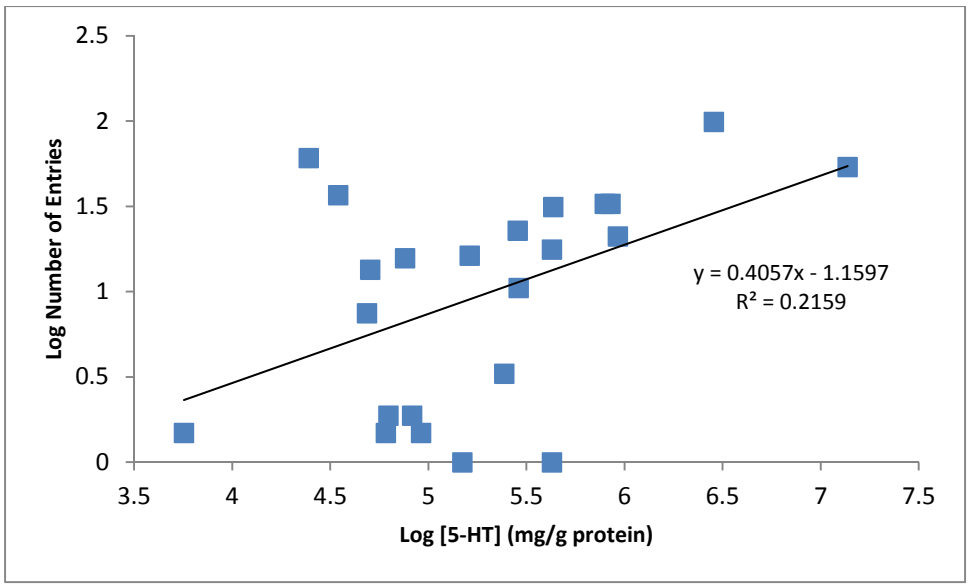

Figure 4.31 Double log plot of fish behavior (number of entries into the light side of the tank) as a function of measured serotonin (5-HT) concentrations after long-term (9 d) diazepam exposure. The equation and correlation coefficient are depicted. p=0.0255.

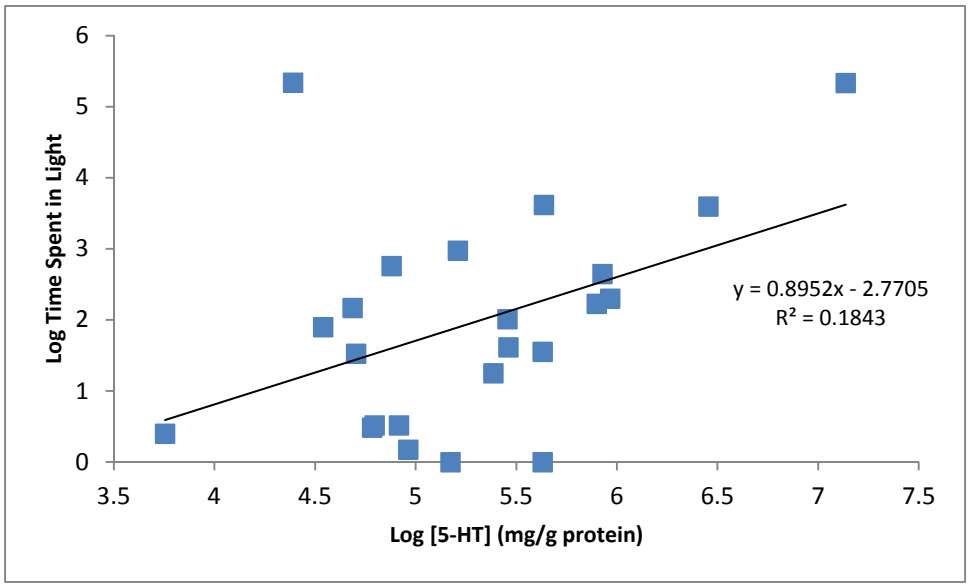

Figure 4.32 Double log plot of fish behavior (time spent on the light side of the tank) as a function of measured serotonin (5-HT) concentrations after long-term (9 d) diazepam exposure. The equation and correlation coefficient are depicted. p=0.0409.

Exposure to anthropogenic contaminants can result in changes in brain chemistry that can then affect an organism's behavior. If this relationship can be understood, scientists will be able to better predict the effects of these contaminants on organisms, leading to better management and regulation practices. However, there have been very few studies that have been able to

successfully correlate organism behavior with brain chemistry. As brain chemistry can be used to predict behavior, I plotted minnow behavior in the light-dark behavioral bioassay as a function of neurotransmitter concentration.

Significant relationships were found between both behavioral endpoints and measured GABA and 5-HIAA concentrations after exposure to acute diazepam as well as between both behavioral endpoints and measured serotonin concentrations after exposure to long-term diazepam (**Figs. 4.27-4.32**). Diazepam was the only drug where a significant relationship between behavioral measures and neurotransmitters was observed. Based on previous research, exposure to these anti-anxiety medications leads to an increase in number of entries to the light side and time spent on the light side, the two behavioral endpoints measured[41,50]. I found that exposure to diazepam resulted in the display of anxiolytic behaviors by minnows on both an acute and long-term scale (**Figs. 4.9** and **4.10**). In addition, exposure to these anti-anxiety medications can result in a decrease in circulating neurotransmitter levels, specifically those that regulate stress and anxiety, like serotonin and GABA[36,37]. Studies investigating the effect of anti-anxiety medications on GABA, another stress-related neurotransmitter, are less reported. I observed decreases in levels of three neurotransmitters after minnows were exposed to diazepam on both an acute and a long-term scale (**Figs. 4.11** and **4.12**). Thus, I hypothesized that as minnow neurotransmitter levels decrease, minnows should enter the light side of the tank more and spend more time on the light side. I found support for the

hypothesis in both GABA and 5-HIAA levels after minnows were exposed to acute diazepam. As GABA and 5-HIAA levels in the brain decreased, minnows made more entries to the light side as well as spent significantly more time in the light. However, although the relationship between these measures was found to be significant in terms of 5-HIAA levels ($p= 0.0274$ and 0.0148, respectively), measured GABA and 5-HIAA levels seem to be weak predictors of minnow entries and time spent in the light, as the respective correlation coefficients 0.148, 0.1617, 0.2023, and 0.2412 suggest. The low correlation coefficients could be the result of a small sample size as well as the inability to extract brains at all timepoints, leading to high variability and a lack of a dose-dependent trend in both the behavioral data as well as the brain data (**Figs. 4.9-4.12**). In contrast, I found a weak, positive relationship between both log number of entries and log time spent in the light and measured serotonin levels after minnows were exposed to diazepam on a long-term scale. As serotonin levels in the brain increased, minnows made more entries to the light side as well as spent more time in the light. These results were the opposite of what was predicted: as serotonin levels in the brain decrease, minnows should enter the light side of the tank more and spend more time on the light side. The relationship was significant despite the low correlation coefficients (R^2=0.2159 and 0.1843). Thus, a change in measured serotonin levels seems to be a weak predictor of minnow anxiety behavior after long-term exposure to diazepam. Serotonin levels measured in minnows exposed to the highest concentration of diazepam were

higher than control minnows (**Fig. 4.12**) which could be an explanation as to why I observed a positive trend between behavioral endpoints and serotonin concentration rather than the predicted negative trend. As diazepam is known to enhance the GABA-ergic system, it comes as no surprise that a weak relationship was found between anxiety behaviors and measured GABA concentrations. Moreover, the weak relationships that were found between neurotransmitters involved in the serotonergic system and diazepam may spark more research on this drug as it has not been suggested that diazepam has any effect on the serotonergic system.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

From this research, I can conclude the following:

- Acute exposure to ethanol, a positive control for this behavioral assay, results in an anxiolytic effect in minnow behavior and brain chemistry at the onset of exposure, but a depressing effect as time goes on due to its nature as a sedative.
- The most definitive results were observed after exposure to diazepam. Exposure to this drug will cause an anxiolytic effect on minnow behavior at high concentrations both acutely (one day) and on a long-term scale (nine days).
- Fluoxetine exposure causes an anxiolytic effect in minnow behavior on an acute time scale, yet results in an overall increase in neurotransmitter concentrations both acutely and on a long-term scale as is supported by previous literature.
- Overall, exposure to anti-anxiety drugs results in a decrease in neurotransmitter levels that may affect predator avoidance behaviors in fathead minnows.
- Acute exposure to copper results in an anxiolytic effect on minnow behavior and a decrease in neurotransmitter levels as time passes and exposure concentrations increase.
- The relationship between behavior and brain chemistry after chemical exposure can be further developed as evidenced by the significant, but

weak relationships that were found between minnow behavior and all measured neurotransmitter levels after exposure to diazepam.

- Results from this study combined with further research will lead to better regulation of contaminants and management of susceptible species.

As stated above, one restriction of the experimental setup was the small sample size, which led to variability among replicates. A larger sample size would offer the possibility of lower variability and more significant trends in the data as well as allow one to tease out the correlation between brain chemistry and behavior in minnows after exposure to contaminants. A larger sample size would also allow brains to be extracted and analyzed after every timepoint during exposure. In addition, the light-dark behavioral bioassay that was used in my experiments was developed for zebrafish. Future exposures using this assay should be performed with zebrafish in order to understand some of the possible species differences. Finally, there is a lack of research concerning the chronic effect of anti-anxiety medications on aquatic organisms. Future studies should be aimed at determining the effects of these medications on aquatic organisms on a long-term scale. Longer exposure times are more relevant as aquatic organisms are more likely to be exposed to sub-lethal doses of these drugs for an extended period of time rather than to acute doses. These studies will provide researchers with additional insight on the fate and metabolism of these drugs in the aquatic environment. Once scientists know more about how these drugs may affect aquatic organisms in the long-term, better management

practices and regulations may be passed for the use and disposal of anti-anxiety medications and other anthropogenic contaminants, leading to a healthier and safer environment for humans.

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